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Applicant:	Collins et al.
Serial No.:	10/783,020
Confirmation No.:	3753
Filed:	February 23, 2004
For:	BIFIDOBACTERIUM IN THE TREATMENT OF INFLAMMATORY DISEASE
Examiner:	V. Afremova
Art Unit:	1651

CERTIFICATE OF MAILING UNDER 37 C.F.R §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to MAIL STOP AMENDMENT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the [illegible] day of June, 2006.

[signature]

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

1. I, Liam O'Mahony, Ph.D., am a microbiologist and immunologist with extensive training and experience in the fields of gastrointestinal immunology; probiotic cultures, including fundamental analysis and therapeutic applications; molecular mechanisms of probiotic actions; biochemistry, physiology and genetics of commensal bacterial flora; and clinical efficacy of probiotic consumption. A copy of my Curriculum Vitae is attached as Exhibit 1.

2. I am a Senior Research Scientist in the Department of Medicine, University College Cork (UCC) and Principal Investigator, Alimentary Pharmabiotic Centre of University College Cork (APC). In addition, I am the chief Immunologist in Alimentary Health Ltd. (AH). AH is a campus-based specialty biotechnology company founded in 1999 to commercialize output of

research from the UCC/BioResearch Ireland (BRI) probiotic research program. UCC is a shareholder in AH. The UCC Probiotic Group and AH, as the Group's corporate partner, secured funding from Science Foundation Ireland; the Alimentary Pharmabiotic Centre (see) was formed as a result. I receive salary from UCC and AH. My research funding is derived from government grants and industry contracts, the latter primarily with AH. My research activities are carried out with UCC, both in APC and AH laboratories.

3. I have reviewed the above-identified patent application, the pending claims, and the Office Action dated December 6, 2005.

4. As indicated at pages 6 - 8 of the Office Action, the Examiner rejected claims 56-62 and 72-88 as anticipated by or obvious over US 6.077,504 (Cavaliere et al.). The Examiner concludes (page 7) that Cavaliere et al. anticipates Applicant's claimed invention because Cavaliere et al. "teaches identical bacterial strain(s) and compositions with these strains wherein the strains belong to the genus of *Bifidobacterium* including strains *Bifidobacterium longum* ATCC 15707 and *Bifidobacterium infantis* ATCC 15697 which are characterized by identical essential features and/or effects including immunomodulation and protection from pathogens as the claimed strain/composition." Further, the Examiner states that even if the claimed strain is not identical to the strains of Cavaliere et al., the differences between that which is disclosed and that which is claimed are so slight that the referenced microorganisms likely inherently possess the same characteristics as the claimed strain. The Examiner concludes that the claimed strain and formulations with the strain would have been obvious to those of ordinary skill in the art.

5. I am providing results of work conducted in my laboratory by Dr. Barbara Sheil (Department of Medicine, UCC) that clearly show that the strains described by Cavaliere et al are markedly different from Applicant's strain (NCIMB 41003, also referred to as Bif 35624). The referenced strains and Applicant's strain differ in a key feature: their immunomodulatory effects. Results of this work are provided in Exhibit 2, entitled "Peripheral Blood Mononuclear

Cell Response to Bifidobacteria." Presented there are results of a peripheral blood mononuclear cell (PBMC) cytokine stimulation assay (for an example of this method, see O'Mahony et al., Clinical & Experimental Immunology 1998). This assay was carried out to compare the effects of the Cavaliere et al. strains and Applicant's claimed strain on cytokine production when the PBMCs are co-incubated with each strain. This assay is useful in assessing the differences among strains because it permits assessment and comparison of the production of pro-inflammatory cytokines and anti-inflammatory cytokines. As shown in Figure 1, stimulation of an anti-inflammatory cytokine, IL-10, by Applicant's strain NCIMB 41003 (Bif 35624) is greater than stimulation of IL-10 by either strain described by Cavaliere et al. (ATCC 15707 and ATCC 15697). Stimulation of IL-10 by NCIMB 41003 (Bif 35624) is significantly greater than stimulation of IL-10 by ATCC 15707 ($p<0.05$). As shown in Figure 2, stimulation of the pro-inflammatory cytokine IL-12 by ATCC 15707 is greater than stimulation of IL-12 by NCIMB 41003 (Bif 35624) ($p<0.05$). In fact, ATCC 15707 stimulates IL-12 production and NCIMB (Bif 35624) does not. Clearly, the strains of Cavaliere et al. and Applicant's strain NCIMB 41003 (Bif 35624) are different in their effects on anti-inflammatory and pro-inflammatory cytokine production.

Further proof of the different immunomodulatory effects of the Cavaliere et al. strains and NCIMB 41003 (Bif 35624) is presented in Figure 4 of Exhibit 2, which illustrates the difference in their respective effects on production of the pro-inflammatory cytokine, TNF-α. Stimulation of TNF-α production by NCIMB 41003 (Bif 35624) is significantly less than stimulation by either of the Cavaliere et al. strains ($p<0.05$ in each case). As shown, both ATCC 15707 and ATCC 15697 stimulate TNF-α in PMBCs. However, NCIMB (Bif 35624) has only a slight effect on TNF-α production. The two strains of Cavaliere et al. are clearly different from NCIMB 41003 in terms of this important immunomodulatory effect.

Yet further proof of the differences between the Cavaliere et al. strains and NCIMB 41003 (Bif 35624) is presented in Figure 4 of Exhibit 2. As shown, the ratio of anti-inflammatory cytokine (IL-10) to pro-inflammatory cytokine (TNF-α) is significantly greater for NCIMB 41003 (Bif 35624) than for either ATCC 15707 or ATCC 15697. A bacterial strain

useful in the treatment of individuals in whom there is a cytokine imbalance (an imbalance in the ratio of anti-inflammatory cytokines to pro-inflammatory cytokines) induces high levels of anti-inflammatory cytokines, but not of pro-inflammatory cytokines. It is a bacterial strain with a high anti-inflammatory to pro-inflammatory cytokine ratio. Individuals with IBS exhibit a cytokine imbalance. I and co-workers have shown that following consumption of NCIMB (Bif 35624), there was a significant improvement in symptoms in IBS patients and the imbalance in anti-inflammatory: pro-inflammatory cytokine ratio was normalised. See, O'Mahony et al., *Gastroenterology* 2005; copy provided with this Amendment. As described in the cited paper, at baseline, patients with IBS demonstrated an abnormal IL-10/IL-12 ratio, indicative of a pro-inflammatory, Th1 state. This ratio was normalized by *B. infantis* 35624 feeding alone, supporting an immune-modulating role for *B. infantis* 35624. Strains with a low anti-inflammatory to pro-inflammatory cytokine ratio--such as those described by Cavaliere et al.--would not be expected to protect against inflammatory conditions or to improve the symptoms (or be useful in the treatment of) patients with IBS. They could, instead, worsen IBS symptoms, since both strains induce significant secretion of the pro-inflammatory cytokine TNF-α and, further, ATCC 15707 does not induce sufficient amounts of IL-10, an anti-inflammatory cytokine.

6. Further evidence of differences between the strains of Cavaliere et al. and NCIMB (Bif 35624) results from assessment of their genomes. Subsequent work carried out by AH scientists Dr. John MacSharry and Graham Sherlock showed that sequences present in genomic DNA of NCIMB (Bif 35624) were not present in either ATCC 15707 or ATCC 15697. See Exhibit 3, which is a gel that shows results of that work. Lane 1: molecular weight standards for reference purposes. (a) Lanes 2-4: primers for a specific region within the NCIMB (Bif 35624) genome were used on all three strains. However, PCR product (approx. 500 base pairs in size) is evident only in the NCIMB (Bif 35624) lane, showing tht ATCC 15707 and ATCC 15697 do not contain the sequence recognized in NCIMB (Bif 35624). (b) Lanes 5-7: PCR primers using primers for repetitive sequences within the genome was carried out on all three strains. Each band represents

one repetitive sequence, which is further evidence that the genome of NCIMB (Bif 35624) is markedly different from that of ATCC 15707 and ATCC 15697.

7. The Examiner asserts that even if the claimed strain UCC 35624 is not identical to the strains of Cavaliere et al., the differences between Applicant's strain and those of Cavaliere et al. are so slight that the strains of Cavaliere et al. likely inherently have the same characteristics as the characteristics of the claimed strain. The Examiner concludes that this is the case "... particularly in view of the similar characteristics which they have been shown to share such as assignment to the same genus and capability of producing immunomodulatory and antimicrobial effects." (Office action, pages 7-8). As discussed above, the strains of Cavaliere et al. and NCIMB 41003 (Bif 35624) are markedly different in their immunomodulatory effects.

In support of her conclusion, the Examiner also points to the fact that the strains of Cavaliere et al. and Applicant's claimed strain are assigned to the same genus. Assignment of microorganisms to the genus *Bifidobacterium* is based on the fact that they share the common characteristics of being anaerobic, gram-positive, irregular or branched rod-shaped bacteria found in the intestine. Classification into the genus is not made with reference to (does not reflect) immunomodulatory characteristics, which are not known for most members of the genus and, as shown by Applicant's assessments, differ markedly among strains. The genus *Bifidobacterium* encompasses many species (at least 30 or more characterized species) and, further, innumerable strains. It is known to those in the field that the characteristics of individual strains of *Bifidobacterium* are determined by many influences, such as the source from which they are obtained, the location within the gastrointestinal tract from which they are derived, and other factors, such as the diet, antibiotic intake and stress experienced by the individual from whom they are obtained. The strains of Cavaliere et al. are both of fecal origin (See, for example, Scardovi V. in Bergey's Manual of Systemic Bacteriology, page 1424: Type strain ATCC 15707 (E 194b from feces of an adult human (Reuter 1971) and Type strain ATCC 15697 (S12 from feces of a human infant (Reuter 1971)); copy provided with the Amendment. Thus, strains ATCC 15707 and ATCC 15697 represent the luminal contents of the distal large bowel (colon).

In contrast, Applicant's strain NCIMB 41003 (Bif 35624) was adherent to the healthy gastrointestinal tract of an individual with no associated pathology.

8. The Examiner has also rejected claims 56-62 and 72-88 as anticipated by or as obvious over Chen et al. (U.S. Patent No. 6,368,591), which describes *Bifidobacterium longum* strain 6-1. It is not correct, as the Examiner appears to conclude, that the strain of Chen et al. is identical to NCIMB 41003 because strain 6-1 and NCIMB 41003 belong to the same genus (*Bifidobacterium)* and are "thus, characterized by identical essential features and/or effects." (Office action, page 9) As discussed in item 7 above, there are many species (at least 30 or more characterized species) encompassed within the genus *Bifidobacterium* and innumerable strains. The characteristics of individual strains are known to be determined by many influences, such as the source from which they are obtained, the location within the gastrointestinal tract from which they are derived, and other factors, such as the diet, antibiotic intake and stress experienced by the individual from whom they are obtained. As also discussed in item 7, Applicant has shown that *Bifidobacterium* strains differ in their characteristics.

I provide here (Exhibit 4) additional evidence of differences between the *B. longum* 6-1 strain of Chen et al. and Applicant's NCIMB 41003 (referred to in Table 1.0 of Exhibit 4 as *B. infantis* 35624). Physicochemical characteristics of *B. longum* 6-1 are shown in Table 1 of U.S. 6,368,591. Physicochemical characteristics of the 6-1 strain and NCIMB 41003 are clearly different, as shown by data presented in the Figure and Table 1.0 of Exhibit 4. Physicochemical characteristics of the 6-1 strain and NCIMB 41003 differ in at least two significant features of their carbohydrate utilization profiles. The carbohydrate utilization profiles of the two microorganisms were assessed under my direction by Joan Gibbon (AH). Chen et al. deposited the *B. longum* 6-1 strain under CCTCC Number M 98003 in the People's Republic of China, China Center for Type Culture Collection, Wuhan, P.R. China. Applicant has been unable to obtain the strain. It is my understanding that if the strain were available from the CCTCC, it would be released only to a recipient within the People's Republic of China and is not available from CCTCC for shipment to recipients outside that country. Therefore, it was not possible to

carry out a direct comparison of the strain of Chen et al. and NCIMB 41003 (*B. infantis* 35624). However, the conditions under which assessment of the physicochemical characteristics of *B. longum* 6-1 was carried out by Chen et al.(resulting in the data presented in Table 1 of the Chen et al. patent) were reproduced in my laboratory and the carbohydrate utilization profile of NCIMB 41003 (*B. infantis* 35624) was determined. In each case, the strain being assessed was cultivated on modified deMann Rogosa Sharpe (MRS) agar plates, in which the chief carbon source normally included (dextrose) was replaced with the sugar whose utilization was being assessed. As shown in the Figure and in Table 1.0, NCIMB (*B. infantis* 35624) grew on all of the sugars, except fructose; it grew on media containing each of the five sugars listed in Table 1.0 (glucose, lactose, mannose, maltose and sucrose). In contrast, the *B. longum* 6-1 strain of Chen et al. showed no growth on media containing either sucrose or mannose. Clearly, the two strains differ in important physicochemical characteristics. For example, differences in carbohydrate fermentation--as seen here--mean that the genomes of the two bacteria are different. The genes required for utilization of sucrose and mannose are found and functional within the genome of NCIMB (*B. infantis* 35624), but not within the genome of *B. longum* 6-1.

9. The Examiner concludes that the Chen et al. patent discloses the strain belonging to the genus *Bifidobacterium* including strain *B. Longum* strain 6-1 and that the 6-1 strain "produces immunomodulatory effects upon oral consumption or regulates immunologic function, decreases abnormal level of cytokine expression (col. 14, lines 43-60). See Office action, page 8. However, Chen et al. describe results of administering only a microbe composition, which includes at least three microorganisms, one of which is *B. Longum* strain 6-1. There is no discussion of giving *B. Longum* strain 6-1 alone (in the absence of the two additional microorganisms) and, thus, there is no evidence in the Chen et al. patent of the characteristics (such as the immunomodulatory effects) of *B. Longum* strain 6-1 alone (assessed in the absence of at least two additional microorganisms). The teachings of the Chen et al. patent do not support the Examiner's description of the characteristics of *B. Longum* strain 6-1.

Art Unit 1644

Serial No. 10/354,447
Conf. No. 8673

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above application and any patent or application related thereto.

Liam O'Mahony, Ph.D.

5th June 2006

Date

Curriculum Vitae.

NAME: Liam O'Mahony
ADDRESS: Alimentary Pharmabiotic Centre, 5th Floor Biosciences Building, University College Cork, National University of Ireland, Cork, Ireland.
CURRENT TITLE: Principal Investigator, Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland

EDUCATION:

1994	BSc (1H Microbiology) University College Cork, Cork, Ireland
1998	PhD (Immunology) Trinity College Dublin, Dublin, Ireland

PROFESSIONAL EXPERIENCE

Oct 2003 – to date	Principal Investigator, Alimentary Pharmabiotic Centre, UCC
Jan 2001 – Sep 2003	Senior Research Scientist, Dept. Medicine, UCC
Jun – Dec 2000	Six month sabbatical, UCLA, USA
Jan 1998 – Jun 2000	Postdoctoral research scientist, Dept. Microbiology, UCC
Sep 1994 – Jan 1998	PhD Graduate Student, TCD

RESEARCH INTERESTS

- Gastrointestinal immunology
- Dendritic cell biology
- Clinical applications for immuno-therapy
- Molecular mechanisms of bacterial-host communication
- Biochemistry, physiology and genetics of commensal bacterial flora
- Clinical efficacy of probiotic consumption

RESEARCH AWARDS

2001-2004	HRB Grant "The Interaction Between Dendritic Cells and Regulatory T-Cells In The Host Immune Response To Bacterial And Self-Antigens In Inflammatory Bowel Disease" - £80,000.
2004-2007	HRB Grant "Molecular mechanisms underpinning probiotic attenuation of T cell mediated colitis" – E165,000.
2006	GSK Grant "Murine models for assessment of T cell trafficking" – E65,000

MANUSCRIPT REVIEWER FOR JOURNALS

Gut; Inflammatory Bowel Diseases; European Journal of Gastroenterology & Hepatology; American Journal of Gastroenterology; Clinical and Diagnostic Laboratory; Immunology; Immunology Letters; Paediatric Research

PUBLICATIONS:

16 Research papers
7 Book chapters
11 Patent applications

SELECTED PUBLICATION EXAMPLES:

Sheil B, Macsharry J, O'callaghan L, O'riordan A, Waters A, Morgan J, Collins JK, O'Mahony L, Shanahan F. Role of interleukin (IL-10) in probiotic-mediated immune

modulation: an assessment in wild-type and IL-10 knock-out mice. Clin Exp Immunol. 2006 May;144(2):273-80.

O'Mahony L, O'Callaghan L, McCarthy J, Shilling D, Scully P, Sibartie S, Kavanagh E, Kirwan WO, Redmond HP, Collins JK, Shanahan F. Differential cytokine response from dendritic cells to commensal and pathogenic bacteria in different lymphoid compartments in humans. Am J Physiol Gastrointest Liver Physiol. 2006 Apr;290(4):G839-45.

O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology. 2005 Mar;128(3):541-51.

Sheil B, McCarthy J, O'Mahony L, Bennett MW, Ryan P, Fitzgibbon JJ, Kiely B, Collins JK, Shanahan F. Is the mucosal route of administration essential for probiotic function? Subcutaneous administration is associated with attenuation of murine colitis and arthritis. Gut. 2004 May;53(5):694-700.

McCarthy J, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK, Shanahan F. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. Gut. 2003 Jul;52(7):975-80.

POSTGRADUATE EDUCATION PROGRAMMES

I have co-supervised 6 PhD post-graduate students (4 of whom have completed their studies), 2 MSc post-graduate students and 5 MD research fellows (3 of whom have completed their studies).

INTERACTION WITH INDUSTRY

Consultation scientist to industry:

1999 – to date	Alimentary Health Ltd
2000 – to date	Procter & Gamble Company
2001 – to date	IAMS
2003 – to date	Mead Johnson

Confidential

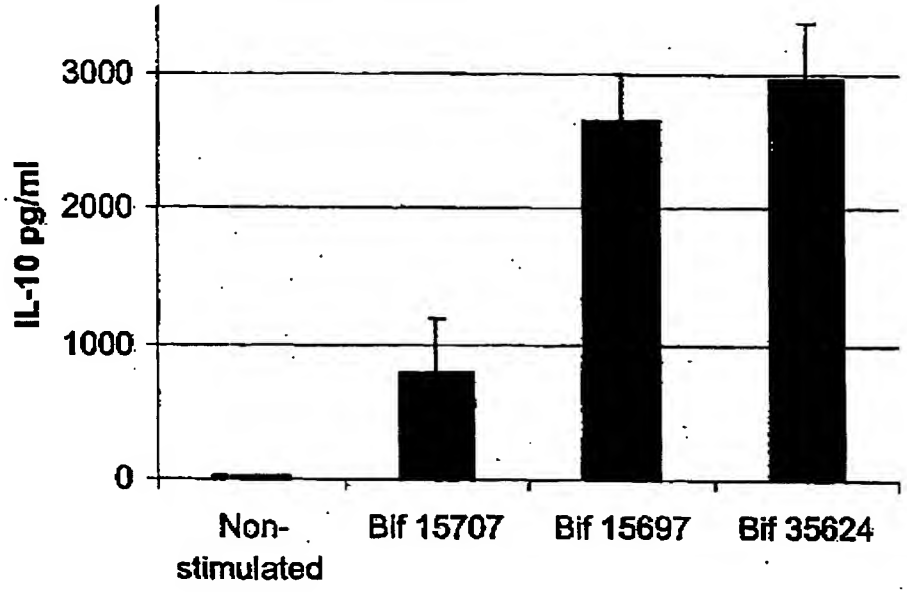
Peripheral Blood Mononuclear Cell Response to Bifidobacteria

A PBMC cytokine stimulation assay was performed to compare the bifidobacterial strains disclosed in US 6,077,504 to *Bifidobacterium infantis* 35624

Liam O'Mahony, PhD

Exhibit 2

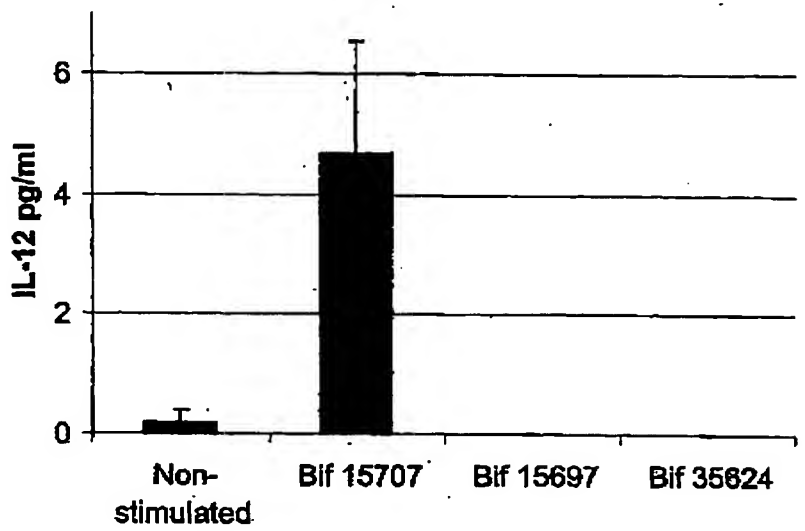
Confidential



Bif 35624 stimulation of IL-10 is significantly different to Bif 15707 stimulation

Liam O'Mahony, PhD

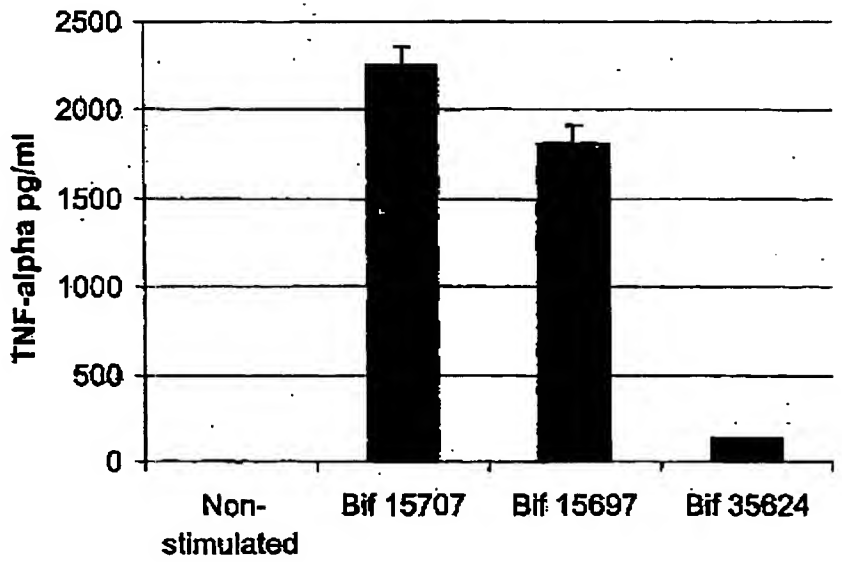
Figure 1



Bif 35624 stimulation of IL-12 is significantly different to Bif 15707 stimulation

Figure 2

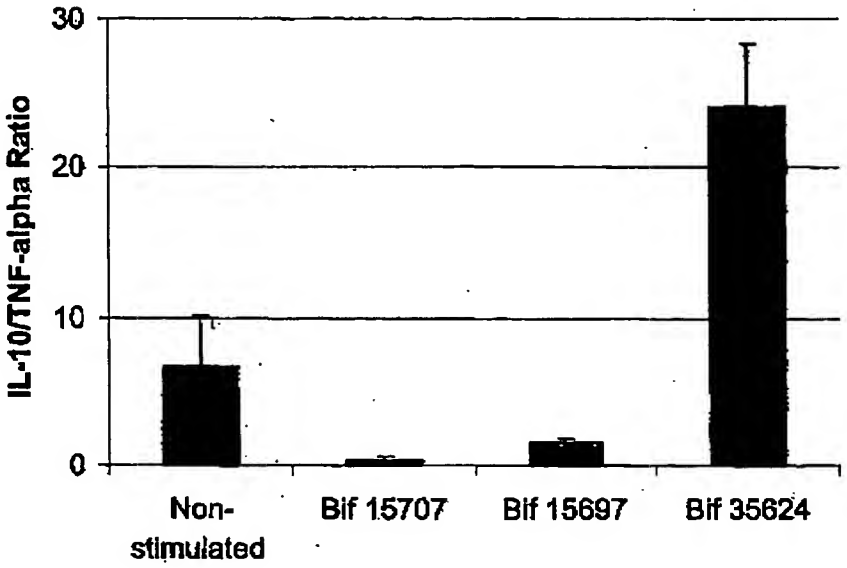
Liam O'Mahony, PhD



Bif 35624 stimulation of TNF-α is significantly different to Bif 15707 and Bif 15697 stimulation

Figure 3

Liam O'Mahony, PhD



Bif 35624 IL-10/TNF-α ratio is significantly different to Bif 15707 and Bif 15697 IL-10/TNF-α ratio

Figure 4

Liam O'Mahony, PhD

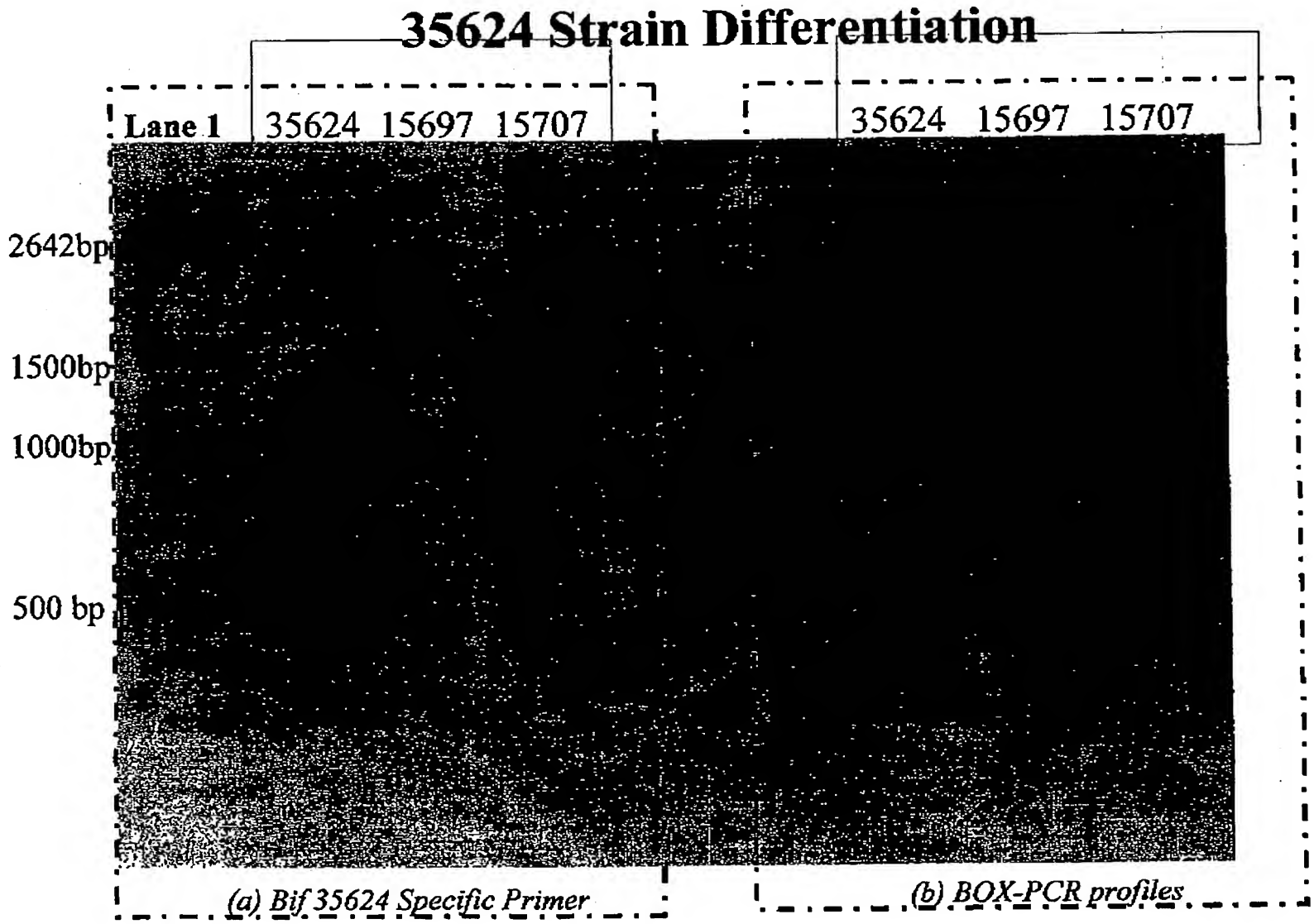
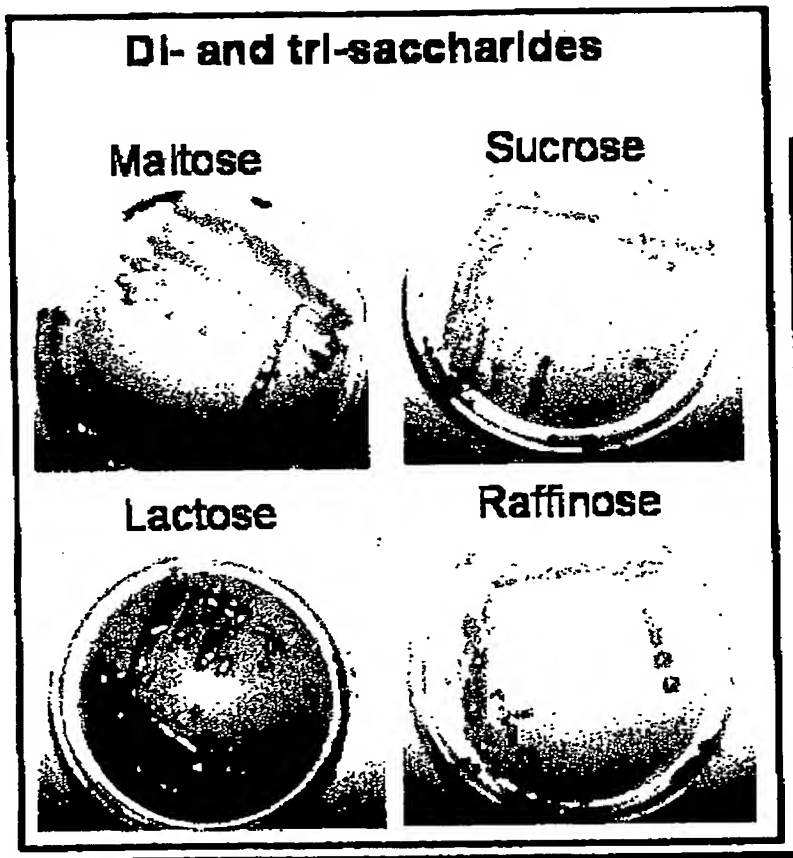
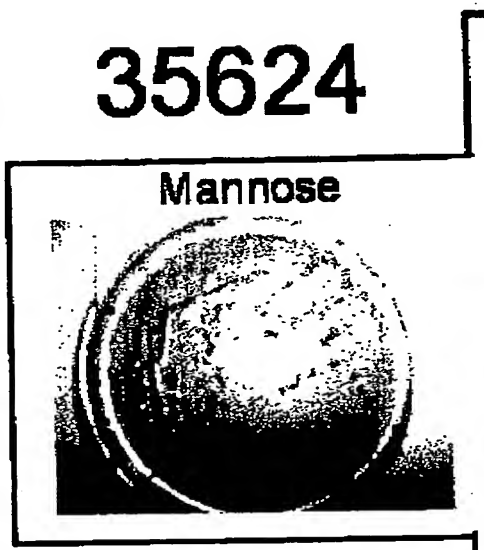

35624 Strain Differentiation
Lane 1
35624 15697 15707
35624 15697 15707
2642bp
1500bp
1000bp
500 bp
(a) Bif 35624 Specific Primer
(b) BOX-PCR profiles

Exhibit 3

35624





mMRS agar plates
MRS with differential carbon sources and supplemented with cysteine

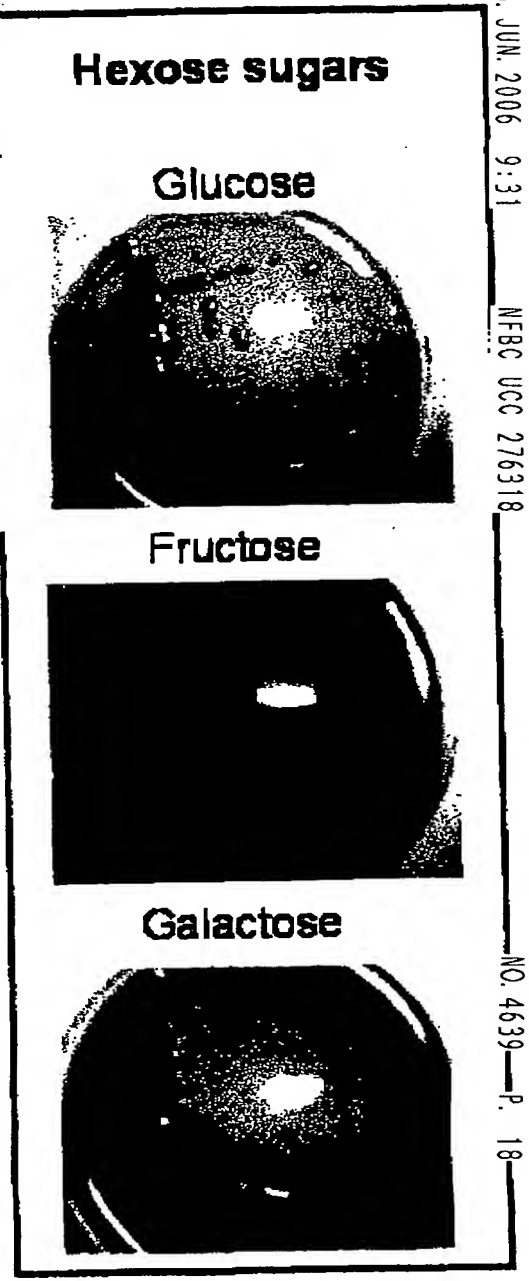


Exhibit 4

Table 1.0

Comparison of Physiochemical characteristics of *B.longum* 6-1 and *B.infantis* 35624

Strain Characteristics	***B.longum* 6-1**	***B.infantis* 35624**
Gram Stain	+	+
Motility	-	-
Milk coagulation	+	+
CHO Fermentation:		
Glucose	+	+
Lactose	+	+
Mannose	-	+
Maltose	+	+
Sucrose	-	+
45°C anaerobic culture	-	-
45°C aerobic culture	-	-

Exhibit 4
Table 1.0

Host-Bacterial Mutualism in the Human Intestine

Fredrik Bäckhed,* Ruth E. Ley,* Justin L. Sonnenburg, Daniel A. Peterson, Jeffrey I. Gordon†

The distal human intestine represents an anaerobic bioreactor programmed with an enormous population of bacteria, dominated by relatively few divisions that are highly diverse at the strain/subspecies level. This microbiota and its collective genomes (microbiome) provide us with genetic and metabolic attributes we have not been required to evolve on our own, including the ability to harvest otherwise inaccessible nutrients. New studies are revealing how the gut microbiota has coevolved with us and how it manipulates and complements our biology in ways that are mutually beneficial. We are also starting to understand how certain keystone members of the microbiota operate to maintain the stability and functional adaptability of this microbial organ.

The adult human intestine is home to an almost inconceivable number of microorganisms. The size of the population—up to 100 trillion—far exceeds that of all other microbial communities associated with the body's surfaces and is ~10 times greater than the total number of our somatic and germ cells (*1*). Thus, it seems appropriate to view ourselves as a composite of many species and our genetic landscape as an amalgam of genes embedded in our *Homo sapiens* genome and in the genomes of our affiliated microbial partners (the microbiome).

Our gut microbiota can be pictured as a microbial organ placed within a host organ: It is composed of different cell lineages with a capacity to communicate with one another and the host; it consumes, stores, and redistributes energy; it mediates physiologically important chemical transformations; and it can maintain and repair itself through self-replication. The gut microbiome, which may contain ≥100 times the number of genes in our genome, endows us with functional features that we have not had to evolve ourselves.

Our relationship with components of this microbiota is often described as commensal (one partner benefits and the other is apparently unaffected) as opposed to mutualistic (both partners experience increased fitness). However, use of the term commensal generally reflects our lack of knowledge, or at least an agnostic (noncommittal) attitude about the contributions of most citizens of this microbial society to our own fitness or the fitness of other community members.

The guts of ruminants and termites are well-studied examples of bioreactors "programmed" with anaerobic bacteria charged with the task of breaking down ingested polysaccharides, the most abundant biological polymer on our planet, and fermenting the resulting monosaccharide soup to short-chain fatty acids. In these mutualistic relationships, the hosts gain carbon and energy, and their microbes are provided with a rich buffet of glycans and a protected anoxic environment (*2*). Our distal intestine is also an anaerobic bioreactor that harbors the majority of our gut microorganisms; they degrade a varied menu of otherwise indigestible polysaccharides, including plant-derived pectin, cellulose, hemicellulose, and resistant starches.

Microbiologists from Louis Pasteur and Ilya Mechnikov to present-day scientists have emphasized the importance of understanding the contributions of this microbiota to human health (and disease). Experimental and computational tools are now in hand to comprehensively characterize the nature of microbial diversity in the gut, the genomic features of its keystone members, the operating principles that underlie the nutrient foraging and sharing behaviors of these organisms, the mechanisms that ensure the adaptability and robustness of this system, and the physiological benefits we accrue from this mutualistic relationship. This Review aims to illustrate these points and highlight some future challenges for the field.

Microbial Diversity in the Human Gut Bioreactor

The adult human gastrointestinal (GI) tract contains all three domains of life—bacteria, archaea, and eukarya. Bacteria living in the human gut achieve the highest cell densities recorded for any ecosystem (*3*). Nonetheless, diversity at the division level (superkingdom or deep evolutionary lineage) is among the lowest (*4*); only 8 of the 55 known bacterial divisions have been identified to date (Fig. 1A), and of these, 5 are rare. The divisions that dominate—the Cytophaga-Flavobacterium-Bacteroides (CFB) (e.g., the genus *Bacteroides*) and the Firmicutes (e.g., the genera *Clostridium* and *Eubacterium*)—each comprise ~30% of bacteria in feces and the mucus overlying the intestinal epithelium. Proteobacteria are common but usually not dominant (*5*). In comparison, soil (the terrestrial biosphere's GI tract, where degradation of organic matter occurs) can contain 20 or more bacterial divisions (*6*).

Our knowledge of the composition of the adult gut microbiota stems from culture-based studies (*7*), and more recently from culture-independent molecular phylogenetic approaches based on sequencing bacterial ribosomal RNA (16*S* rRNA) genes. Of the >200,000 rRNA gene sequences currently in GenBank, only 1822 are annotated as being derived from the human gut; 1689 represent uncultured bacteria. rRNA sequences can be clustered into relatedness groups based on their percent sequence identity. Cutoffs of 95 and 98% identity are used commonly to delimit genera and species, respectively. Although these values are somewhat arbitrary and the terms "genus" and "species" are not precisely defined for microbes, we use them here to frame a view of human gut microbial ecology. When the sequences (n = 495 greater than 900 base pairs) are clustered into species, and a diversity estimate model is applied, a value of ~800 species is obtained (Fig. 2). If the analysis is adjusted to estimate strain number (unique sequence types), a value of >7000 is obtained (Fig. 2). Thus, the gut microbiota, which appears to be tremendously diverse at the strain and subspecies level, can be visualized as a grove of eight palm trees (divisions) with deeply divergent lineages represented by the fan(s) of closely related bacteria at the very top of each tree trunk.

Diversity present in the GI tract appears to be the result of strong host selection and coevolution. For example, members of the CFB division that are predominantly associated with mammals appear to be the most derived (i.e., farthest away from the common ancestor of the division), indicating that they underwent accelerated evolution once they adopted a mutualistic lifestyle. Moreover, a survey of GenBank reveals that several subgroups in CFB are distributed among different mammalian species (Fig. 1B), suggesting that the CFB-mammal symbiosis is ancient and that distinct subgroups coevolved with their hosts.

Center for Genome Sciences, Washington University School of Medicine, St. Louis, MO 63108, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: jgordon@molecool.wustl.edu

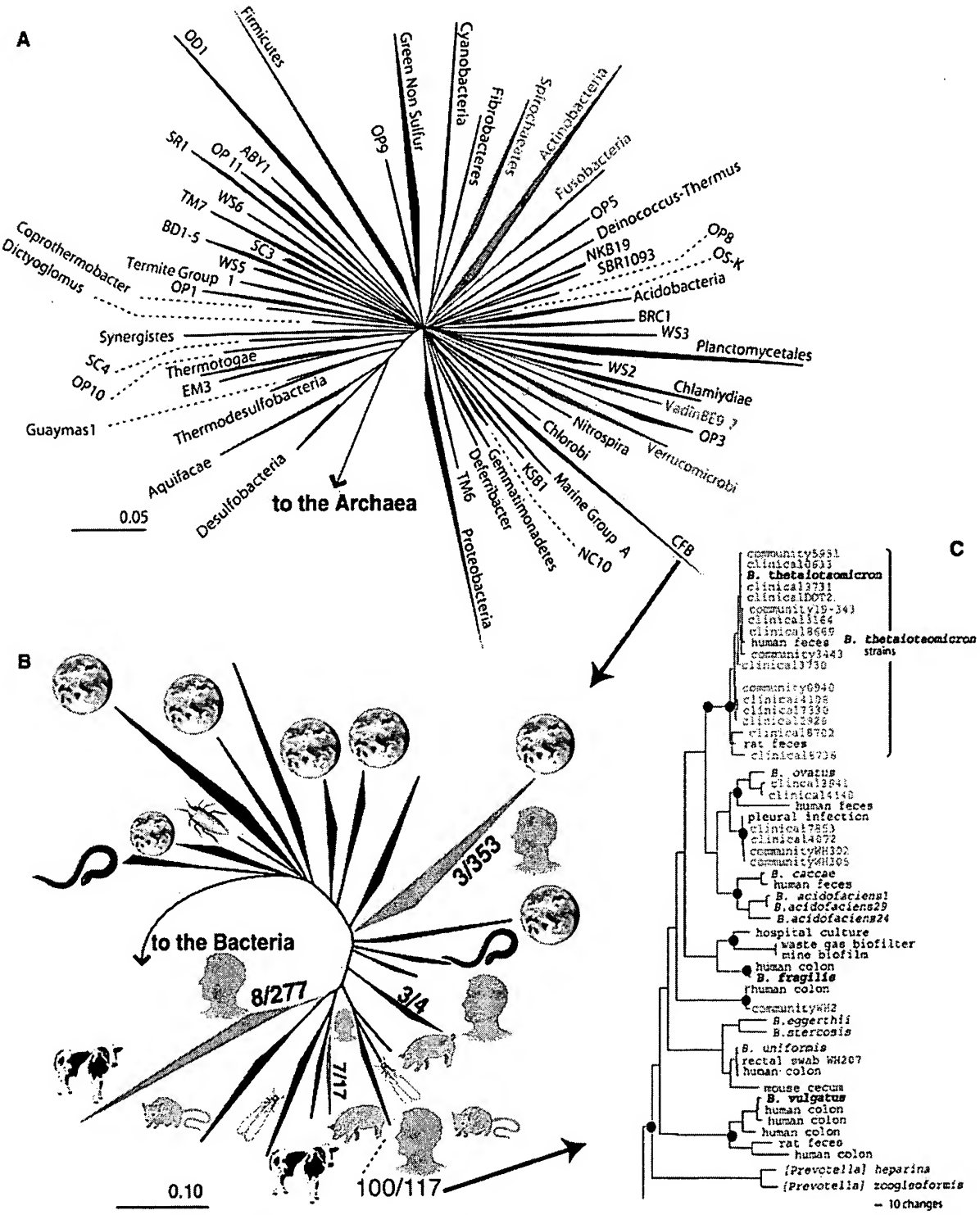


Fig. 1. Representation of the diversity of bacteria in the human intestine. (A) Phylogenetic tree of the domain bacteria based on 8903 representative 16S rRNA gene sequences. Wedges represent divisions (superkingdoms): Those numerically abundant in the human gut are red, rare divisions are green, and undetected are black. Wedge length is a measure of evolutionary distance from the common ancestor. (B) Phylogenetic tree of the CFB division based on 1561 sequences from GenBank (>900 nucleotides) and their ecological context. Wedges, major subgroups of CFB; symbols, source of the sequences [Earth, environmental; cow, ruminants; rodent, rat and/or mouse; person, human GI tract; others are termite, cockroach, worm (including hydrothermal), and pig]. Ratios are the number of sequences represented in the human gut relative to the total number in the subgroup; red, yellow, and black indicate majority, minority, and absence of sequences represented in human GI tract, respectively. (C) Phylogenetic (parsimony) tree of *Bacteroides*. Strains classified as *B. thetaiotaomicron* based on phenotype are in red; 16S rRNA analysis did not confirm this classification for all strains. *Bacteroides* spp. with sequenced genomes are in bold. Black circles indicate nodes with high (>70%) bootstrap support (*41*). Scale bars indicate the degree of diversity (evolutionary distance) within a division or subgroup [(A) and (B), respectively] in terms of the fraction of the 16S rRNA nucleotides that differ between member sequences; in (C), the evolutionary distance between organisms is read along branch lengths, where scale indicates number of changes in 16S rRNA nucleotide composition.

The structure and composition of the gut microbiota reflect natural selection at two levels: at the microbial level, where lifestyle strategies (e.g., growth rate and substrate utilization patterns) affect the fitness of individual bacteria in a competitive ensemble; and at the host level, where suboptimal functionality of the microbial ensemble can reduce host fitness. Microbial consortia whose integrated activities result in a cost to the host will result in fewer hosts, thereby causing loss of their own habitat. Conversely, microbial consortia that promote host fitness will create more habitats. Thus, the diversity found within the human GI tract, namely, a few divisions represented by very tight clusters of related bacteria, may reflect strong host selection for specific bacteria whose emergent collective behavior is beneficial to the host. This hypothesis has two important implications: (i) A mechanism exists to promote cooperation, and (ii) the structure promotes functional stability of the gut ecosystem.

To benefit the host, bacteria must be organized in a trophic structure (food web) that aids in breaking down nutrients and provides the host with energetic substrates. Cooperative behavior that imposes a cost to the individual while benefiting the community can emerge within groups of bacteria (*8*) and can be maintained by group selection as long as consortia are isolated and new consortia form periodically (e.g., new GI tracts). Furthermore, selection must act simultaneously at multiple levels of biological organization (*9*). These criteria are met in the human GI tract where new acts of colonization occur at birth, with a small founding population of noncheaters from the mother, and selection occurs both at the microbial and host level.

Diversity is generally thought to be desirable for ecosystem stability (*10*). One important way diversity can confer resilience is through a wide repertoire of responses to stress [referred to as the insurance hypothesis (*11*)]. In man-made anaerobic bioreactors used to treat wastewater (a system analogous to the gut but where no host selection occurs), rates of substrate degradation can remain constant, whereas bacterial populations fluctuate chaotically as a result of blooms of subpopulations (*12*). Functional redundancy in the microbial community ensures that key processes are unaffected by such changes in diversity (*13*). By contrast, in the human gut, populations are remarkably stable within individuals (*14*), implying that mechanisms exist to suppress blooms of subpopulations and/or to promote the abundance of desirable bacteria. A study of adult monozygotic twins living apart and their marital partners has emphasized the potential dominance of host genotype over diet in determining microbial composition of the gut bioreactor (*15*). The role of the immune system in defining diversity and suppressing subpopulation blooms remains to be defined. One likely mediator of bacterial selection is secretory immunoglobulin A (*16*).

The human gut is faced with a paradox: How can functional redundancy be maintained in a system with low diversity (few divisions of bacteria), and how can such a system withstand selective sweeps in the form of, for example, phage attacks? [The estimated 1200 viral genotypes in human feces (*17*) suggest that phage attack is a powerful shaper of the gut's microbial genetic landscape (*18*, *19*)]. Enough diversity of genome and transcriptome must be represented at the subspecies level to lend resilience to the gut ecosystem. Ecological studies of macroecosystems have shown that less diversity is required to maintain stability if individual species themselves have a wide repertoire of responses (*11*). In the following section we discuss recent genome-based studies exploring how a presumed keystone bacterial species in our gut is able to adapt to (i) changing dietary conditions in ways that should stabilize the microbiota's food web and (ii) changing immune and phage selective pressures in ways that should stabilize the ecosystem.

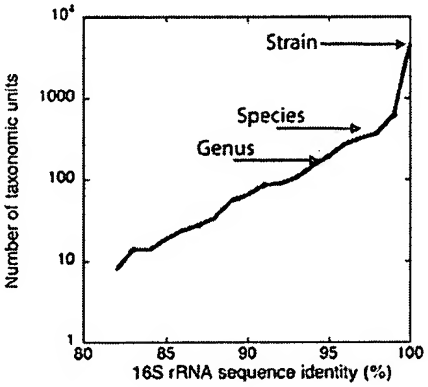


Fig. 2. Taxon richness estimates for bacteria in the human GI tract. Taxon richness estimates (*41*) for varying levels of 16*S* rRNA sequence identity, ranging from below the "genus" level (95% identity), to the "species" level (98% identity), to the strain level (unique sequences). Estimates are based on sequences available in GenBank, annotated as derived from the human GI tract, after alignment and clustering into taxonomic units ranging from 80 to 100% identity (*41*).

Bacteroides thetaiotaomicron—A Highly Adaptive Glycophile

Bacteroides thetaiotaomicron is a prominent mutualist in the distal intestinal habitat of adult humans. It is a very successful glycophile whose prodigious capacity for digesting otherwise indigestible dietary polysaccharides is reflected in the fully sequenced 6.3-Mb genome of the type strain (ATCC 29148; originally isolated from the feces of a healthy adult human) (*20*). Its "glycobiome" contains the largest ensemble of genes involved in acquiring and metabolizing carbohydrates yet reported for a sequenced bacterium, including 163 paralogs of two outer membrane proteins (SusC and SusD) that bind and import starch (*21*), 226 predicted glycoside hydrolases, and 15 polysaccharide lyases (*22*). By contrast, our 2.85-Gb genome only contains 98 known or putative glycoside hydrolases and is deficient in the enzyme activities required for degradation of xylan-, pectin-, and arabinose-containing polysaccharides that are common components of dietary fiber [we have one predicted enzyme versus 64 in *B. thetaiotaomicron* (table S1)].

The carbohydrate foraging behavior of *B. thetaiotaomicron* has been characterized during its residency in the distal intestines (ceca) of gnotobiotic mice colonized exclusively with this anaerobe (*23*). Scanning electron microscopy studies of the intestines of mice maintained on a standard high-polysaccharide chow diet, containing xylose, galactose, arabinose, and glucose as its principal monosaccharide components, revealed communities of bacteria assembled on small undigested or partially digested food particles, shed elements of the mucus gel layer, and exfoliated epithelial lining cells (*23*). Whole-genome transcriptional profiling of *B. thetaiotaomicron* (*23*) disclosed that this diet was associated with selective up-regulation of a subset of SusC and SusD paralogs, a subset of glycoside hydrolases (e.g., xylanases, arabinosidases, and pectate lyase), as well as genes encoding enzymes involved in delivering the products of mannose, galactose, and glucose to the glycolytic pathway and arabinose and xylose to the pentose phosphate pathway. In contrast, a simple sugar (glucose and sucrose) diet devoid of polysaccharides led to increased expression of a different subset of SusC and SusD paralogs, glycoside hydrolases involved in retrieving carbohydrates from mucus glycans, as well as enzymes that remove modifications that make these host glycans otherwise resistant to degradation (O-acetylation of sialic acids and sulfation of glycosoaminoglycans) (*23*).

These findings provide insights about how functional diversity and adaptability are achieved by a prominent member of the human colonic microbiota (Fig. 3). Dining occurs on particulate nutrient scaffolds (food particles, shed mucus, and/or exfoliated epithelial cells). For a bacterium such as *B. thetaiotaomicron*, which lacks adhesive organelles, seating at the "dining table" is determined in part by the repertoire of glycan-specific outer membrane–binding proteins it produces, and this repertoire is itself shaped by the menu of available glycans (*23*). Attachment to nutrient platforms helps avoid washout from the intestinal bioreactor, in much the same way as dense, well-settling, granular biofilms help oppose elimination from engineered (man-made) an-

aerobic upflow bioreactors (*24*). Attachment also presumably increases the efficiency of oligo- and monosaccharide harvest by adaptively expressed bacterial glycoside hydrolases and their subsequent distribution to other members of the microbiota whose niche overlaps that of *B. thetaiotaomicron*. In this conceptualization, microbial nutrient metabolism along the length of the intestine is a summation of myriad selfish and syntrophic relationships expressed by inhabitants of these nutrient platforms. Diversity in these microhabitats and mutualistic cooperation among their component species (including the degree to which sanctions must be applied against cheats) are reflections of a dynamic interplay between the available nutrient foundation and the degree of flexible foraging (niche breadth) expressed by microhabitat residents. *Bacteroides* spp., such as *B. thetaiotaomicron*, impart stability to the gut ecosystem by having the capacity to turn to host polysaccharides when dietary polysaccharides become scarce. The highly variable outer chain structures of mucus and epithelial cell surface glycans are influenced by host genotype and by microbial regulation of host glycosyltransferase gene expression. Coevolution of host glycan diversity and a large collection of microbial glycoside hydrolases that are regulated by nutrient availability provides insurance that the "system" (microbiota and host) can rapidly and efficiently respond to changes in the diet, and maximize energy harvest, without having to undergo substantial changes in species composition. Rather than minimizing genome size, a keystone species such as *B. thetaiotaomicron* has evolved an elaborate and sizable genome that can mobilize functionally diverse adaptive responses.

Diet-associated changes in the glycan foraging behavior of *B. thetaiotaomicron* are also accompanied by changes in expression of its capsular polysaccharide synthesis loci (*CPS*), indicating that *B. thetaiotaomicron* is able to change its carbohydrate surface depending upon the nutrient (glycan) environment. This could be part of a strategy for evading an adaptive immune response. Whole-genome genotyping studies of *B. thetaiotaomicron* isolates, with the use of GeneChips designed from the sequenced genome of the type strain, disclose that their *CPS* loci differ, whereas their housekeeping genes are conserved (*25*). Because selective sweeps are most likely to come from the immune system and phages, both of which respond to surface structures, the associated genes are likely to be the most diverse in the genome. Accordingly, *B. thetaiotaomicron* has a remarkable apparatus for altering its genome content. The sequenced type strain contains a plasmid, 63 transposases, 43 integrases, and four homologs of a conjugative transposon (*20*). Gene transfer and mutation mechanisms endow strains of bacterial species with the (genetic) versatility necessary to withstand selective sweeps that would eradicate more clonal populations (*26*).

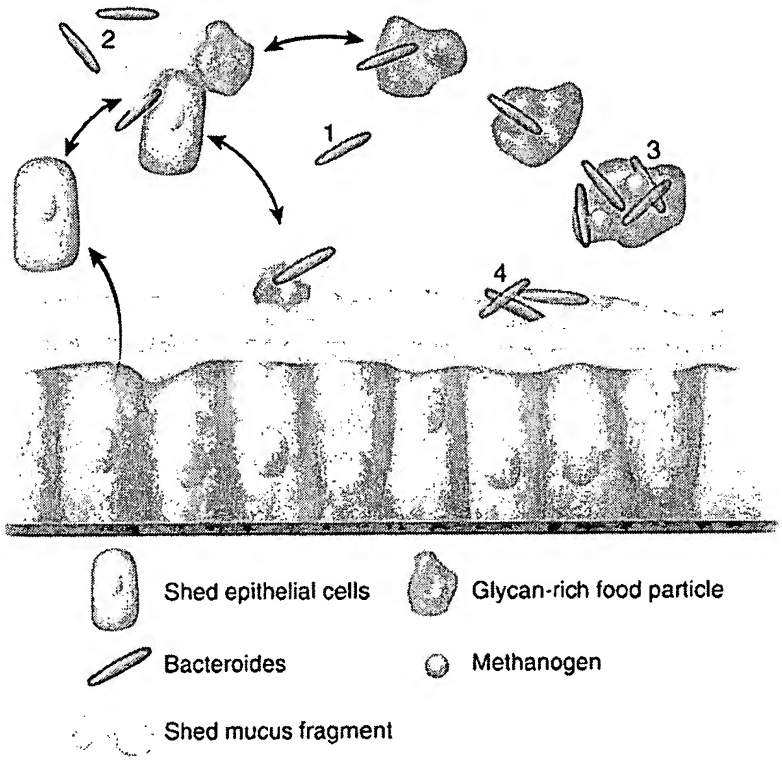


Fig. 3. Lessons about adapative foraging for glycans obtained from *B. thetaiotaomicron*. (1) *B. thetaiotaomicron* does not have adhesive organelles. Without outer membrane polysaccharide-binding protein-mediated attachment to glycan-rich nutrient platforms, it is at risk for being washed out from the intestinal bioreactor. Substrate access is limited under these conditions. (2) Small nutrient platforms are composed of undigested or partially digested food particles (e.g., dietary fiber), shed host epithelial cells, and/or mucus fragments. These platform elements may be in dynamic equilibrium with one another and with the mucus layer overlying the intestinal epithelium. Microbial fermentation of otherwise indigestible polysaccharides in these platforms is made possible by induced expression of substrate-appropriate sets of bacterial polysaccharide-binding proteins and glycoside hydrolases. (3) Mesophilic methanogens drive carbohydrate utilization by removing products of fermentation (H_2 and CO_2 are converted to methane), thereby improving the overall efficiency of energy extraction from polysaccharides. (4) When dietary polysaccharides are scarce, *B. thetaiotaomicron* turns to host mucus by deploying a different set of polysaccharide binding proteins and glycoside hydrolases. This adaptive foraging reflects the coevolved functional versatility of *B. thetaiotaomicron*'s glycobiome and the structural diversity of the host's mucus glycans.

The Gut Microbiota as a "Host" Factor That Influences Energy Storage

Comparisons of mice raised without exposure to any microorganisms [Germ-Free (GF)] with those that have acquired a microbiota since birth [Conventionally Raised (CONV-R)] have led to the identification of numerous effects of indigenous microbes on host biology (table S2), including energy balance. Young adult CONV-R animals have 40% more total body fat than their GF counterparts fed the same polysaccharide-rich diet, even though CONV-R animals consume less chow per day (*27*). This observation might seem paradoxical at first but can be explained by the fact that the gut microbiota allows energy to be salvaged from otherwise indigestible dietary polysaccharides (*28*). "Conventionalization" of adult GF mice with cecal contents harvested from CONV-R donors increases body fat content to levels equivalent to those of CONV-R animals (*27*). The increase reflects adipocyte hypertrophy rather than hyperplasia and is notable for its rapidity and sustainability (*27*).

The mutualistic nature of the host-bacterial relationship is underscored by mechanisms that underlie this fat-storage phenotype. Colonization increases glucose uptake in the host intestine and produces substantial elevations in serum glucose and insulin (*27*), both of which stimulate hepatic lipogenesis through their effects on two basic helix-loop-helix/leucine zipper transcription factors—ChREBP and SREBP-1c (*27*, *29*). Short-chain fatty acids, generated by microbial fermentation, also induce lipogenesis (*30*). Triglycerides exported by the liver into the circulation are taken up by adipocytes through a lipoprotein lipase (LPL)–mediated process. The microbiota suppresses intestinal epithelial expression of a

circulating LPL inhibitor, fasting-induced adipose factor (Fiaf, also known as angiopoietin-like protein-4) (*27*). Comparisons of GF and conventionalized wild-type and *Fiaf*$^{-/-}$ mice established Fiaf as a physiologically important regulator of LPL activity in vivo and a key modulator of the microbiota-induced increase in fat storage (*27*).

The caloric density of food items is portrayed as a fixed value on package labels. However, it seems reasonable to postulate that caloric value varies between individual "consumers" according to the composition and operation (e.g., transit time) of their intestinal bioreactors, and that the microbiota influences their energy balance. Relatively high-efficiency bioreactors would promote energy storage (obesity), whereas lower efficiency reactors would promote leanness (efficiency is defined in this case as the energy-harvesting and storage-promoting potential of an individual's microbiota relative to the ingested diet).

The idea that individual variations in bioreactor efficiencies may be a significant variable in the energy balance equation is supported by several observations. First, individual variations in the composition of the microbiota occur and are influenced by host genotype (*15*). Second, small but chronic differences between energy intake and expenditure can, in principle, produce major changes in body composition [e.g., if energy balance is +12 kcal/day, >0.45 kg of fat could be gained per year if there are no compensatory responses by the host; this is the average weight increase experienced by Americans from age 25 to 55 (*31*)]. Third, the microbiota is a substantial consumer of energy. One group estimated that individuals on a "British Diet" must ferment 50 to 65 g of hexose sugars daily to obtain the energy required to replace the 15 to 20 g (dry weight) of bacteria they excrete per day (*32*).

These considerations emphasize the need to assess the representation of species with large capacities for processing dietary polysaccharides, such as *Bacteroides*, in lean versus morbidly obese individuals, and in cohorts of obese individuals before, during, and after weight reduction achieved by high-polysaccharide/low-fat versus high-fat/low-polysaccharide diets [or by bariatric (gastric bypass) surgery]. The results, coupled with coincident assessments of energy extraction from the diet, should provide a proof-of-concept test of whether differences in the composition of the microbiota are associated with differences in gut bioreactor efficiency (and predisposition to obesity).

Lessons that have been learned by environmental engineers who study how to optimize the efficiency of man-made anaerobic bioreactors (table S3) suggest that these enumeration studies should also include members of archaea. Thermodynamics dictates that the energy obtained from substrate conversions will be higher if low concentrations of products are maintained (*33*, *34*). In the human gut, methanogenic archaea provide the last microbial link in the metabolic chain of polysaccharide processing. Bacteria degrade polysaccharides to short-chain fatty acids, CO_2, and hydrogen gas. Methanogens lower the partial pressure of hydrogen by generating methane, and thereby may increase microbial fermentation rates. Defining the representation of mesophilic methanogens in the colonic microbiota of individuals, sequencing their genomes [as we are currently doing with *Methanobrevibacter smithii*, a prevalent isolate from the human colon (*35*)], and characterizing archaeal-bacterial syntrophy in simplified gnotobiotic mouse models consuming different diets should provide a starting point for defining the role of archaea in shaping the functional diversity, stability, and beneficial contributions of our distal gut microbiota. Devising ways for manipulating archaeal populations may provide a novel way for intentionally altering our energy balance.

Looking to the Future

A comprehensive 16*S* rRNA sequence–based (bacterial and archaeal) enumeration of the microbiotas of selected humans, representing different ethnic groups, living in similar or distinct milieus, would provide an invaluable database for studying normal and diseased populations (*36*). The concept of using the microbiota as a biomarker of impending or fully manifest diseases within or outside of the GI tract and for monitoring responses to therapeutic interventions needs to be explored.

Several groups are embarking on metagenome sequencing projects to define gene content in the human gut microbiome. If we view ourselves as being a composite of many species, this represents a logical continuation of the Human Genome Project. A complementary approach to metagenomic analysis is to determine genome-level diversity among bacterial populations belonging to a specific genus or species residing within a defined gut habitat of a single individual or a few individuals. Members of *Bacteroides* provide a natural experiment for examining the impact of habitat on genome content since they have yet to be encountered in any environment other than animal GI tracts. Figure 1C illustrates how a collection of just 29 isolates phenotyped as *B. thetaiotaomicron* provided a broad range of 16*S* rRNA sequences, including several new species. We are close to producing finished genome sequences for two prominent members of the colonic microbiota, *B. vulgatus* and *B. distasonis* (*37*). *B. fragilis*, a less prominent member, has recently been sequenced (*38*, *39*). The results will allow us to ask how evolutionary history relates to genome content and what constitutes a minimal *Bacteroides* genome.

We also need to obtain a direct view of how the metabolites originating from the microbiome influence host physiology. This will be a formidable task, requiring new techniques for measuring metabolites generated by single and defined collections of symbionts during growth under defined nutrient conditions in single-vessel chemostats, in more elaborate mechanical models of the human gut, and in vivo after colonization of specified habitats of the intestines of gnotobiotic mice. The results should help formulate and direct hypothesis-based investigations of the microbiota's "metabolome" in humans.

Databases that connect molecular data with ecosystem parameters are still rare (*40*). A human intestinal microbiome database is needed to organize genomic, transcriptomic, and metabolomic data obtained from this complex natural microbial community, and would provide a substrate for generating testable hypotheses.

Finally, just as microbiotas have coevolved with their animal hosts, this field must coevolve with its academic hosts and their ability to devise innovative ways of assembling interactive interdisciplinary research groups necessary to advance our understanding.

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42. We thank L. Angenent for many helpful discussions. Work cited from the authors' lab is supported by the NIH and NSF. F.B. and J.L.S. are supported by postdoctoral fellowships from the Wenner-Gren and W. M. Keck Foundations, respectively.

Supporting Online Material
www.sciencemag.org/cgi/content/full/307/5717/1915/DC1
Materials and Methods
Tables S1 to S3
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REVIEW

Immunity, Inflammation, and Allergy in the Gut

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The gut immune system has the challenge of responding to pathogens while remaining relatively unresponsive to food antigens and the commensal microflora. In the developed world, this ability appears to be breaking down, with chronic inflammatory diseases of the gut commonplace in the apparent absence of overt infections. In both mouse and man, mutations in genes that control innate immune recognition, adaptive immunity, and epithelial permeability are all associated with gut inflammation. This suggests that perturbing homeostasis between gut antigens and host immunity represents a critical determinant in the development of gut inflammation and allergy.

The gastrointestinal tract is the site where the divergent needs of nutrient absorption and host defense collide: The former requires a large surface area and a thin epithelium that has the potential to compromise host defense. Many infectious diseases involve the gut, and the investment by the gut in protecting itself is evident in the abundant lymphoid tissue and immune cells it harbors. In westernized countries, most infectious diseases of the gut are largely under control, yet gastrointestinal food allergies and idiopathic inflammatory conditions have dramatically increased; in other words, we now have inflammation without infection. Although the reason for this remains unknown, a prevailing notion is that the absence of overt gut infection has upset the balance between the normal bacteria that colonize the healthy gut and the mucosal immune system.

The Gut Epithelial Barrier

The primary cellular barrier of the gut in preventing antigens encountering the immune system is the single layer of gut epithelium, the surface area of which is expanded to the order of 400 m^2, largely because it is formed into millions of fingerlike villi in the small bowel. Each epithelial cell maintains intimate association with its neighbors and seals the surface of the gut with tight junctions. In the upper bowel, the bulk of the antigen exposure comes from diet, whereas in the ileum and colon, the additional antigenic load of an abundant and highly complex commensal microflora exists.

Nevertheless, the gut epithelial barrier does not completely prevent lumenal antigens from entering the tissues. Thus, intact food proteins can be detected in plasma (*1*), and a few gut bacteria can be detected in the mesenteric lymph nodes draining the gut of healthy animals (*2*). Antigens can cross the epithelial surface through breaks in tight junctions, perhaps at villus tips where epithelial cells are shed, or through the follicle-associated epithelium (FAE) that overlies the organized lymphoid tissues of the intestinal wall (*3*). Peyer's patches (PP) in the small bowel are aggregates of lymphoid tissue numbering ~200 in the average adult, although tens of thousands of much smaller individual follicles also line the small bowel and colon. FAE contains specialized epithelial cells termed M cells whose function is to transport lumenal antigens into the dome area of the follicle (*3*) (Fig. 1). Antigen-presenting dendritic cells (DC) also send processes between gut epithelial cells without disturbing tight junction integrity and sample commensal and pathogenic gut bacteria (*4*, *5*). The gut epithelial barrier therefore represents a highly dynamic structure that limits, but does not exclude, antigens from entering the tissues, whereas the immune system constantly samples gut antigens through the FAE and DC processes.

Commensal Bacteria in Epithelial/Immune Cell Function in the Gut

Interaction of commensals with gut epithelium. The gut epithelium itself can also directly sense commensal bacteria and pathogens; integral to this are the mammalian pattern recognition receptors (PRRs), which recognize conserved structures of bacteria and viruses and generally activate pro-inflammatory pathways alerting the host to infection (*6*). Two different classes of PRRs are involved. The Toll-like receptors (TLRs) are usually associated with cell membranes and have an external leucine-rich repeat (LRR) recognition domain and an intracellular interleukin-1 receptor (IL-1R)–like signaling domain (*7*). The nucleotide-binding oligomerization domain (Nod) molecules, Nod1 and Nod2 [also known as CARD4 and CARD15 (caspase activation and recruitment domain)], are present in the cytosol of epithelial cells and immune cells. These proteins also have LRRs at the C terminus, a Nod domain, and CARD domains at the N terminus (*8*). There is abundant evidence that signaling through Nod or TLR activates transcription factor NF-κB, leading to pro-inflammatory gene expression (*7*, *8*)

TLR1 to TLR9 and Nod1 and Nod2 are each expressed by gut epithelial cells (*6*, *9*). Nod1 and Nod2 recognize slightly different muropeptide motifs derived from bacterial peptidoglycans (*6*), which suggests that they sense intracellular infection or attempted bacterial subversion of epithelial cells (*10*). TLRs recognize many different components of bacteria and viruses. For example, TLR4 recognizes

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CLINICAL–ALIMENTARY TRACT

Lactobacillus and *Bifidobacterium* in Irritable Bowel Syndrome: Symptom Responses and Relationship to Cytokine Profiles

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See editorial on page 783.

Background & Aims: The aim of this study was to compare the response of symptoms and cytokine ratios in irritable bowel syndrome (IBS) with ingestion of probiotic preparations containing a lactobacillus or bifidobacterium strain. *Methods:* Seventy-seven subjects with IBS were randomized to receive either *Lactobacillus salivarius* UCC4331 or *Bifidobacterium infantis* 35624, each in a dose of 1×10^{10} live bacterial cells in a malted milk drink, or the malted milk drink alone as placebo for 8 weeks. The cardinal symptoms of IBS were recorded on a daily basis and assessed each week. Quality of life assessment, stool microbiologic studies, and blood sampling for estimation of peripheral blood mononuclear cell release of the cytokines interleukin (IL)-10 and IL-12 were performed at the beginning and at the end of the treatment phase. *Results:* For all symptoms, with the exception of bowel movement frequency and consistency, those randomized to *B infantis* 35624 experienced a greater reduction in symptom scores; composite and individual scores for abdominal pain/discomfort, bloating/distention, and bowel movement difficulty were significantly lower than for placebo for those randomized to *B infantis* 35624 for most weeks of the treatment phase. At baseline, patients with IBS demonstrated an abnormal IL-10/IL-12 ratio, indicative of a proinflammatory, Th-1 state. This ratio was normalized by *B infantis* 35624 feeding alone. *Conclusions: B infantis* 35624 alleviates symptoms in IBS; this symptomatic response was associated with normalization of the ratio of an anti-inflammatory to a proinflammatory cytokine, suggesting an immune-modulating role for this organism, in this disorder.

Irritable bowel syndrome (IBS) is a common functional disorder usually defined by the coexistence of abdominal pain or discomfort and an alteration in bowel habit.[1–3] IBS may lead to impaired social and personal function and can diminish quality of life to a degree usually associated with major organic diseases such as hypertension and diabetes.[4,5] IBS represents a significant therapeutic challenge; currently available therapies provide symptomatic relief at best, and none have been shown to alter the natural history of the disorder.[1,2,6–11] While the precise pathophysiology of IBS remains to be elucidated,[12] dysmotility and altered visceral perception/sensation are currently the most popular hypotheses.[13] More recently, roles for enteric infection and intestinal inflammation have been proposed. Thus, both retrospective and prospective studies have documented the new onset of IBS following bacteriologically confirmed bacterial gastroenteritis[14–21] and others have provided evidence of low-grade mucosal inflammation[22–24] and immune activation[25–27] in patients with IBS. The enteric flora has also been implicated; there has been a suggestion that some patients with IBS may harbor bacterial overgrowth and that their symptoms may be ameliorated by its eradication.[28–31] Despite these observations, our ever-increasing understanding of gut flora-mucosa interactions,[32] and the existence of a significant body of basic research to support a role for inflammatory and immune processes in contributing to enteric neuromuscular dysfunction,[33] the role of lumen-mucosa interactions in IBS remains largely unexplored.

Probiotics, defined as live or attenuated bacteria or bacterial products that confer a significant health benefit to the host,[34] have the potential to provide a clinical tool to explore these interactions. There are several reasons why these agents might, in theory, prove of therapeutic

Abbreviations used in this paper: AUC, area under the curve; IBS, irritable bowel syndrome; IL, interleukin; PBMC, peripheral blood mononuclear cell; VAS, visual analogue scale.

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benefit in IBS. Firstly, many probiotic organisms exert antibacterial and antiviral effects and could thereby prevent or modify the course of postinfective IBS.[35,36] Secondly, probiotics have been demonstrated to exert anti-inflammatory effects at mucosal surfaces[37,38]; by reducing mucosal inflammation, probiotics could decrease immune-mediated activation of enteric motor and sensory neurons and modify neural traffic between the gut and the central nervous system. Thirdly, probiotics could alter the composition of the gut flora. While the status of the gut flora in IBS remains a source of some controversy,[12,39,40] probiotic-related changes in the enteric flora could directly (through the augmentation of commensal lactobacilli or bifidobacteria or the elimination of pathogens) or indirectly (through a reduction in either pathogen-related inflammation or bacterial fermentation[41]) influence gut function. Finally, probiotics could alter the volume and/or composition of stool and gas[42] or increase intestinal mucus secretion,[43] effects that could influence intestinal handling of its contents and thus modulate symptoms such as constipation and diarrhea.

A small number of studies have evaluated the response of IBS to probiotic preparations; while results between studies are difficult to compare because of differences in study design, probiotic dose, and strain, there has been some, but by no means consistent, evidence of symptom improvement.[44–53] The overall impact of probiotics in IBS remains unclear.[54–58] Several of these studies have involved either lactobacilli or bifidobacteria, although none have involved a direct comparison of these strains.[55]

Patients and Methods

Study Population

Patients were recruited from gastroenterology clinics at Cork University Hospital and by direct advertisement on the university campus and in a local newspaper. Individuals aged between 18 and 75 years who satisfied Rome II criteria for the diagnosis of IBS[3] and in whom organic gastrointestinal diseases, including inflammatory bowel disease, and clinically significant systemic diseases had been excluded were considered for inclusion in the study. Pregnant women, individuals with known lactose intolerance or immunodeficiency, and individuals who had undergone any abdominal surgery, with the exception of hernia repair and appendectomy, were excluded.

Trial Protocol

Each potentially eligible patient was evaluated by a full review of clinical history and performance of a physical examination as well as full blood count, serum chemistry, and quantitative serum immunoglobulin levels. Clinically significant abnormalities in any of the latter test results led to exclusion from randomization. Eligible subjects then entered a 4-week run-in period during which they recorded symptoms, as well as stool frequency and form, each day on a diary card. During this time and throughout the rest of the study, subjects were instructed not to take any medications that could influence gut motor or absorptive function, including laxatives and antidiarrheal agents, as well as any preparation that could alter the enteric flora, including antibiotics and commercially available probiotic preparations.

At the end of the run-in period, subjects were randomized to receive either a lactobacillus or bifidobacterium, each delivered in a dose of 1×10^{10} live bacterial cells in a malted milk drink, or the malted milk drink alone as placebo. All preparations were identical in color, taste, and consistency. Randomization was performed by picking a card from a pack of prerandomized identical cards in the presence of a study coordinator; all other investigators, as well as patients, remained blinded to the randomization process until completion of the trial. Subjects were instructed to ingest the preparation once a day, in the morning, for 8 weeks and record symptoms and stool characteristics on a daily basis throughout the study period. Compliance was assessed by direct questioning at clinic visits and by fecal flora analysis. On completion of the 8-week treatment phase, subjects continued to complete the daily symptom cards for a further 4-week washout period while off all therapy.

Probiotic Preparations

The probiotic preparations used in this study, *Lactobacillus salivarius* subspecies *salivarius* UCC4331 and *Bifidobacterium infantis* 35624, were originally isolated from the ileocecal region of an adult human undergoing reconstructive surgery. These strains were selected on the basis of the following probiotic properties: being of human origin, nonpathogenic, and resistant to intestinal acid and bile; demonstrating an ability to adhere to human epithelial cells; and demonstrating an ability to temporarily colonize and be metabolically active within the human gastrointestinal tract.[59,60] Furthermore, these organisms have been previously shown in volunteer studies to survive transit through the gastrointestinal tract, to be free of side effects, and to demonstrate anti-inflammatory activity in a number of models.[60,61] *L salivarius* UCC4331 was cultured in de Man/Rogosa/Sharp broth (Oxoid, Basingstoke, United Kingdom) at 37°C in an anaerobic environment for 24 hours. *B infantis* 35624 was cultured in de Man/Rogosa/Sharp broth enriched with cysteine at 37°C in an anaerobic environment for 48 hours.

Assessments

Throughout the entire study, subjects were seen on a weekly basis and diary cards collected. The following 3 cardinal IBS symptom clusters were assessed: (1) abdominal pain or discomfort, (2) bloating or distention, and (3) bowel movement difficulty. The latter could reflect either difficulty with

evacuation (ie, straining or a sense of incomplete evacuation) or urgency. Each symptom was evaluated using both an ordinal scale (Likert scale; maximum score, 7) and a 10-cm visual analogue scale (VAS; maximum score, 10).[62] A composite score, comprised of the sums of the 3 cardinal symptoms (pain/discomfort, bloating/distention, and bowel movement difficulty scores) was also calculated for each patient (maximum score: Likert scale, 21; VAS, 30).

Bowel movement frequency was recorded as number per day, and consistency was evaluated using the Bristol Stool Scale.[63]

Quality of life was assessed by administration of an IBS-specific questionnaire developed and validated by Drossman et al[64] at the time of randomization and at the end of the treatment and washout periods. The following 8 domains were assessed on each occasion in each patient: dysphoria, interference with activity, body image, health worry, food avoidance, social reaction, sexual function, and impact on relationships.

Blood samples for blood count, serum chemistry, and quantitative immunoglobulin levels were obtained at initial evaluation and at the end of the study and analyzed using standard laboratory methods.

Stool samples for fecal flora analysis were obtained at randomization and at the end of the treatment phase. Spontaneous rifampicin-resistant variants of both probiotic strains were isolated before initiation of this study to facilitate the differentiation of these bacteria from all other lactobacilli and bifidobacteria. Representative fecal suspensions were serially diluted and plated on de Man/Rogosa/Sharp agar containing rifampicin or de Man/Rogosa/Sharp agar containing cysteine and rifampicin to enumerate *L salivarius* UCC4331 or *B infantis* 35624, respectively.

Peripheral blood samples from patients with IBS was obtained both before and after treatment for cytokine levels and compared with that obtained from a group of age- and sex-matched healthy volunteers (n = 20). Peripheral blood samples were taken directly into sterile EDTA-containing Vacutainers (Econo-med, Long Sutton, United Kingdom). Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation[65] and resuspended at 1×10^6 cells/mL in complete media/Dulbecco's modified Eagle medium containing 25 mmol/L glucose, 10% fetal calf serum, 1% nonessential amino acids, 50 U/mL penicillin, and 50 μg/mL streptomycin (Invitrogen, Paisley, Scotland). These mononuclear cells are termed peripheral blood mononuclear cells (PBMCs). PBMCs were incubated, nonstimulated, for 72 hours at 37°C in a 5% CO_2 humidified atmosphere. Nonstimulated PBMC cytokine production reflects the cytokine milieu from which the PBMCs were originally isolated. Cell-free supernatants were stored frozen at −70°C and analyzed for cytokine levels in batches. Interleukin (IL)-10 and IL-12p40 cytokine levels were measured using enzyme-linked immunosorbent assays (R&D Systems, Abington, United Kingdom).

Statistical Methods

All data were collected and analyzed independently of the investigators, who did not have access to the data or to its analysis until the latter had been completed. All of the efficacy analyses were summarized on data from all evaluative subjects and analyzed on an intention-to-treat basis. Baseline and demographic data were tested for balance across treatment groups using 1-way analysis of variance, χ^2 test, or Fisher exact test, as appropriate.

For each of the IBS symptoms and the composite score, efficacy data were analyzed in 2 ways. First, weekly individual symptom scores and composite scores were analyzed using a repeated-measures analysis of covariance model, with fixed effects for treatment and for the mean week 1 (baseline) symptom or composite score, as applicable. The variance/covariance matrix in this model was assumed to have compound symmetry. Second, to compare scores over the entire treatment phase, an area-under-the curve (AUC) analysis was performed for each symptom and the composite score. Symptom scores were first averaged within each week for each subject. AUCs were then calculated for each subject by using week 1 to week 8 scores in the treatment phase. AUCs were then analyzed using analysis of covariance using the baseline score at week 2 of the run-in phase as covariate and treatment as the factor in the model. Tukey's method for adjustment for multiple treatment comparisons was used to obtain the adjusted *P* values for between-treatments comparisons. Both adjusted and unadjusted *P* values were reported.

Quality-of-life measurements were analyzed using analysis of covariance with fixed effects for treatment group and baseline.

Results

Subjects

A total of 80 subjects were enrolled in the study. Two subjects (1 randomized to *B infantis* 35624 and 1 to placebo) were subsequently found to have taken antibiotics from the beginning of the treatment phase and were therefore determined to be nonevaluable. A further 3 subjects dropped out before the treatment phase. Therefore, 75 subjects provided some evaluable data. Of these, 3 subjects took an antibiotic during the course of the study; only data up to the commencement of antibiotic use were deemed to be evaluable. Another subject took a commercially available probiotic preparation during the washout/follow-up period (weeks 8–12); only data up to week 8 were determined to be evaluable for this subject. Four additional subjects failed to complete the washout phase; therefore, 67 subjects satisfactorily completed all phases of the study.

Baseline Characteristics

Among the 75 evaluable subjects, 64% were women and 36% were men. Subjects averaged 44.3 years in age (range, 18–73 years). Sex and age were both balanced across treatment groups. All subjects were white. Classification of subjects at baseline by predominant symptom indicated that 45% were alternators, 28% diarrhea predominant, and 26% constipation predomi-

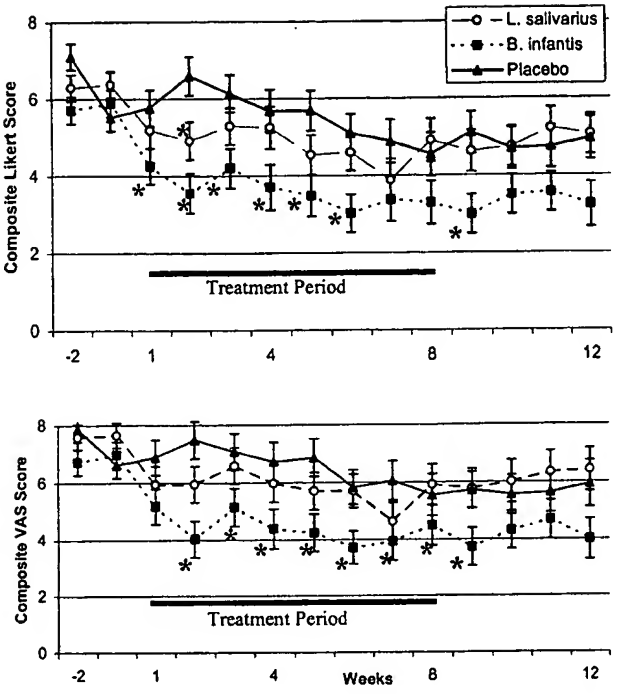

Figure 1. Composite Likert scale and VAS scores. Comparison of the effects of placebo, *L salivarius* UCC4331, and *B infantis* 35624 on a composite score of IBS symptoms. The scores are derived from the sum of scores for abdominal pain/discomfort, bloating/distention, and bowel movement difficulty. Note the significant reduction in composite scores throughout the treatment period and into the washout phase for subjects treated with *B infantis* 35624 but not with *L salivarius* UCC4331 or placebo. $*P < .05$ vs placebo; all comparisons adjusted for any differences in baseline symptom score.

nant. In addition, 25% of the subjects were smokers and 88% drank alcohol. Although subjects were balanced across treatment groups with respect to use of alcohol, there was imbalance detected with regard to smoking status; of the subjects responding, 72% of the *L salivarius*

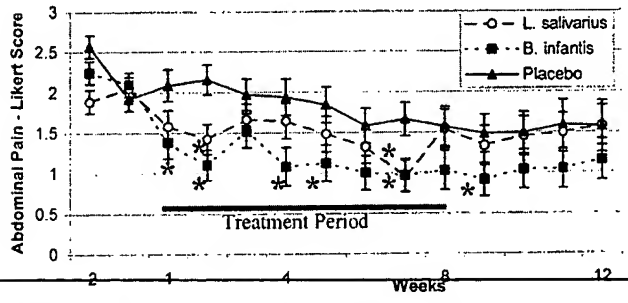

Figure 2. Abdominal pain scores on the Likert scale. Comparison of the effects of placebo, *L salivarius* UCC4331, and *B infantis* 35624 on abdominal pain/discomfort in IBS. Note the significant reduction in pain/discomfort score during most weeks of the treatment phase and into the washout phase among patients treated with *B infantis* 35624; for those randomized to *L salivarius* UCC4331, a significant benefit was evident at week 2 alone. $*P < .05$ vs placebo; all comparisons adjusted for any differences in baseline symptom score.

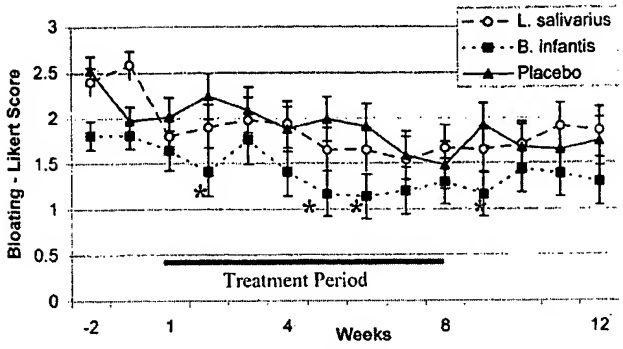

Figure 3. Bloating scores on the Likert scale. Comparison of the effects of placebo, *L salivarius* UCC4331, and *B infantis* 35624 on bloating/distention in IBS. Note the significant reduction in bloating/distention during weeks 2, 5, and 6 for those randomized to *B infantis* 35624; there was no significant benefit for those randomized to *L salivarius* UCC4331. $*P < .05$ vs placebo; all comparisons adjusted for any differences in baseline symptom score.

UCC4331 group did not smoke, compared with 92% of the *B infantis* 35624 group and 58% of the placebo group ($P = .03$).

The 3 treatment groups were not quite balanced for baseline symptom scores. Statistically significant imbalance was detected at the $\alpha = .05$ level for the following baseline scores: abdominal pain/Likert score (week −2), bloating/Likert score (week −2), bloating/Likert score (week −1), bloating/VAS score (week −2), bloating/VAS score (week −1), and the composite Likert score (week −2). In consideration of these baseline differences, efficacy analyses were performed using baseline, as calculated by the average of the week −1 symptom scores, as a covariate.

Response to Treatment

Figures 1–4 summarize the least-squares means and standard errors for composite score and each of the primary symptom efficacy measurements (abdominal pain/discomfort, bloating/distention, and bowel movement difficulty) according to treatment and for each week of the 8-week treatment period and the 4-week washout period. Because similar results were obtained for all parameters studied using both the Likert scale and the VAS, results for both scales are presented for composite score alone; elsewhere, only the Likert scale results are presented. Table 1 presents results of the AUC analyses.

A comparison of scores for each week showed that subjects treated with *B infantis* 35624 had lower composite scores than those receiving placebo for all weeks in the treatment phase and the entire washout phase. Of the VAS scores for each of these 12 weeks, 10 were significantly lower than those for placebo. The only 2 weeks in which scores were not significantly lower than those for

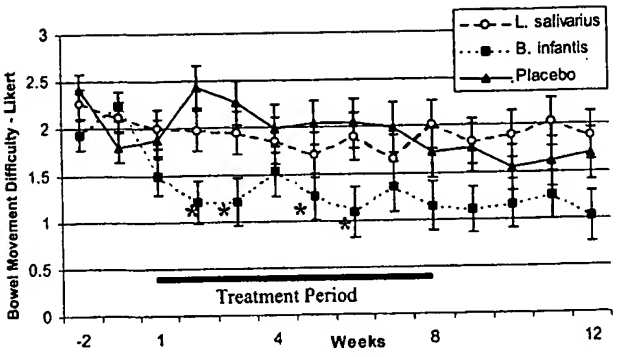

Figure 4. Bowel movement difficulty scores on the Likert scale. Comparison of the effects of placebo, *L salivarius* UCC4331, and *B infantis* 35624 on bowel movement difficulty in IBS. Note the significant reduction in bowel movement difficulty during weeks 2, 3, and 5–7 for those randomized to *B infantis* 35624; there was no significant benefit for those randomized to *L salivarius* UCC4331. *$P < .05$ vs placebo; all comparisons adjusted for any differences in baseline symptom score.

placebo were weeks 10 and 11, during the washout phase (Figure 1). In comparison, those randomized to *L salivarius* UCC4331 achieved a statistically significant reduction in composite symptom score during the second week of the treatment period alone (Figure 1). Furthermore, on at least 1 of the scales, composite scores for those treated with *B infantis* 35624 were significantly lower than for those treated with *L salivarius* UCC4331 during the second, fourth, sixth, and eighth weeks of the treatment phase and for 3 of the 4 weeks of the washout phase (Figure 1).

Comparison of AUCs for the treatment phase showed significantly lower Likert scale and VAS composite scores for the group randomized to *B infantis* 35624 compared with the placebo group, even after controlling for multiple between-treatments comparisons (Table 1).

For each individual symptom, with the notable exceptions of bowel movement frequency and consistency, the group randomized to *B infantis* 35624 experienced a greater reduction in symptom scores during the treatment period (Figures 1–4 and Table 1). Thus, subjects randomized to *B infantis* 35624 achieved lower scores for pain/discomfort (AUC, $P < .05$ vs placebo for unadjusted scores, Table 1; Likert scale scores for individual weeks, $P < .05$ vs placebo for weeks 1, 2, 4, 5, and 7 of the treatment phase and week 1 of the washout phase, Figure 2), bloating/distention (AUC, $P < .07$ vs placebo for unadjusted scores, Table 1; Likert scale scores for individual weeks, $P < .05$ vs placebo for weeks 2, 5, and 6 of the treatment phase, Figure 3), and bowel movement difficulty (AUC, $P < .005$ vs placebo for adjusted scores, Table 1; Likert scale scores for individual weeks, $P < .05$ vs placebo for weeks 2, 3, 5, and 6 of the treatment phase and week 1 of the washout phase, Figure 4). In contrast, the only symptom improvement observed for those randomized to *L salivarius* UCC4331 was an improvement in abdominal pain during weeks 2 and 7 of the treatment phase (Likert scale, $P < .05$, Figure 2); none of the AUC comparisons showed a significant effect for *L salivarius* UCC4331 on an individual symptom (Table 1).

Direct comparisons between the 2 probiotic-treated groups showed significantly lower ($P \leq .05$) scores for bowel movement difficulty for those treated with *B infantis* 35624 (AUC, $P < .05$ *B infantis* 35624 vs placebo or *L salivarius* UCC4331, Table 1; Likert scale scores for individual weeks, $P < .05$ *B infantis* 35624 vs *L salivarius* UCC4331 for weeks 2, 3, and 6 of the treatment phase and weeks 1 and 4 of the washout phase, Figure 4).

The time course of the response to *B infantis* 35624 demonstrated a relatively rapid response; improvement was evident at the end of the first week and reached a maximum by the end of the second week of an 8-week course of therapy (Figures 1 and 4).

Subjects receiving the 3 treatments reported a similar number of bowel movements per week and similar bowel movement consistency scores across all 8 weeks of the treatment period.

Quality of Life

For most domains, quality-of-life scores were numerically lower than those for placebo for the patients randomized to the probiotics but reached statistical significance versus placebo during the treatment phase only for health worry for bifidobacterium (at the .05 level) and dysphoria for lactobacillus (at the .10 level).

Table 1. AUC Analysis of Therapeutic Response

	L salivarius UCC4331	*B infantis* 35624	Placebo
Abdominal pain			
Likert score	8.98 (1.36)	7.78 (1.36)[a]	12.21 (1.85)
VAS score	11.40 (1.82)	9.45 (1.69)[a]	14.92 (2.39)
Bloating			
Likert score	12.61 (1.68)	10.17 (1.67)[b]	14.39 (2.18)
VAS score	15.32 (2.44)	11.66 (2.35)[b]	17.04 (3.14)
Bowel movement difficulty			
Likert score	15.61 (1.85)	7.84 (1.91)[c]	16.79 (2.34)
VAS score	19.71 (2.18)[b]	11.16 (2.14)[c]	24.50 (2.83)
Stool consistency	22.22 (1.66)	25.51 (1.65)	22.98 (2.11)
Composite			
Likert score	34.64 (3.60)	24.56 (3.63)[c]	40.52 (4.68)
VAS score	42.35 (4.93)	30.15 (4.80)[c]	52.14 (6.39)

NOTE. Values are expressed as least-squares mean (SE).
[a] $P < .05$.
[b] $.05 < P < .10$, between-treatments difference without adjustment.
[c] $P < .05$ between-treatments difference after adjustment for multiple comparisons.

PBMC Cytokine Levels

In vitro production of IL-10 and IL-12 by PBMCs was dysregulated at baseline in patients with IBS compared with healthy volunteers (Figure 5). IL-10 levels were lower in patients with IBS (575 ± 108 pg/mL vs 968 ± 220 pg/mL), whereas IL-12 levels were increased in patients with IBS (15 ± 2 pg/mL vs 6 ± 4 pg/mL). The ratio of IL-10/IL-12 levels was significantly different between the 2 groups (IBS, 69 ± 15; healthy volunteers, 176 ± 31; $P = .003$).

Following treatment with *B infantis* 35624, PBMC cytokine levels returned to levels similar to those observed for the healthy volunteer group (Figure 5). In contrast, PBMC cytokine levels did not return to levels observed for healthy volunteers in the subjects with IBS who received *L salivarius* UCC4331 or placebo.

Adverse Events

Four subjects reported adverse events during the study; 1 developed an episode of epistaxis that resolved spontaneously, 1 was hospitalized with unstable angina and another with an episode of chest pain that was attributed to anxiety, and 1 was hospitalized with abdominal pain that was attributed to an exacerbation of IBS and constipation. No clinically significant changes in full blood count, serum chemistry, or serum immunoglobulin levels were recorded in any of the subjects during the study.

Stool Recovery of Probiotics

Growth was observed on rifampicin-containing media with samples obtained from probiotic-treated patients, thus confirming that the probiotics consumed had survived gastrointestinal transit in the patients with IBS. No growth was observed from fecal samples obtained before probiotic feeding or at any point in patients receiving placebo.

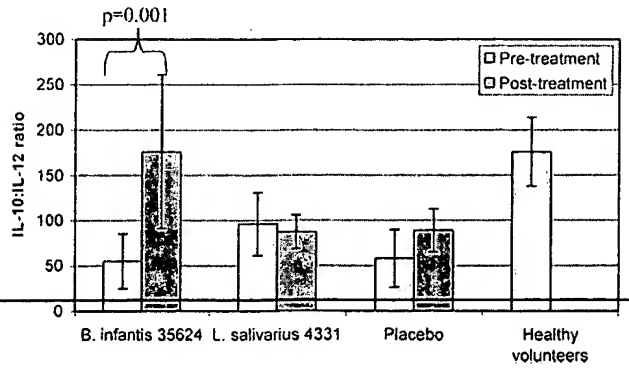

Figure 5. PBMC IL-10/IL-12 ratio. Comparison of PBMC IL-10/IL-12 ratios at baseline and following therapy with placebo, *L salivarius* UCC4331, and *B infantis* 35624 with that of a normal control period. Note the abnormal baseline ratio in subjects with IBS, with a normalization of this ratio following the administration of *B infantis* 35624 alone.

Discussion

In this study, we compared, for the first time, the effects of 2 probiotic strains on symptoms in patients with IBS. We have shown superiority for bifidobacterium over both a lactobacillus and placebo for each of the cardinal symptoms of IBS and for a composite score. These symptomatic benefits were associated with parallel trends in a quality-of-life measure developed specifically for IBS.[64] Furthermore, this therapy was well tolerated and free of significant adverse events. Interestingly, these benefits, in contrast to those observed with 2 newly approved therapies for IBS, namely alosetron[66] and tegaserod,[67,68] were observed independent of any change in stool frequency or form and cannot therefore be attributed to either a laxative or an antidiarrheal effect.

This study is not without its limitations. The small size of the study population may have failed to detect significant effects of either of the probiotics on some symptoms. Furthermore, the study was not powered to detect significant changes in quality of life. Symptoms were assessed on the basis of paper diaries, which are subject to recall bias and are therefore less accurate than electronic diaries. To capture the overall impact of IBS on the subject, we chose to use a composite score comprised of the cardinal features of IBS; an alternative approach to this same issue would have been to use some form of global assessment instrument. Assessments of individual symptoms also permitted the evaluation of the therapies on individual components of IBS. It needs to be stressed, however, that the small size of this study would, if anything, have mitigated against our ability to demonstrate differences. The fact that we did demonstrate efficacy for one probiotic strain over both placebo and another strain further supports the validity of these observations and suggests that the bifidobacterium strain used may have a selective and specific effect in IBS. It should also be noted that the placebo response rate observed in this study was similar to that recorded in many other IBS studies. As evidenced by their symptom scores at entry, the patients studied lay at the mild end of IBS and were more reflective of the type of patients with IBS seen in the community rather than in referral centers. Whether bifidobacterium would be similarly effective in the latter population remains to be seen. We chose to accept all eligible patients with

IBS for this study and did not exclude on the basis of sex or symptom predominance, an approach that has been adopted in studies of the serotonin agonists and antagonists.[66–68] In an attempt to score equally for symptoms related to defecation in a diarrhea- or constipation-predominant subject with IBS, we used the parameter of bowel movement difficulty, which could score for either urgency in the patient with diarrhea, or difficulty with evacuation (straining, incomplete evacuation), which is a symptom complex associated with constipation. Furthermore, the individual treatment groups were too small to permit a meaningful post-hoc subgroup analysis of response based on these or other parameters. The duration of the study was also similar to that of other recent studies but does not permit an assessment of the long-term effects of this therapy. Although this study did not involve a comparison with any other therapeutic modality and the study design differed in some aspects from recent large trials of serotonergic agonists and antagonists, the therapeutic gain observed for bifidobacterium over placebo (20%–25%) is certainly no less than that reported for tegaserod and alosetron (10%–20%).[66–68]

We chose *L salivarius* UCC4331 over several lactobacillus probiotic strains isolated and characterized in our laboratories, based on in vitro activity against several pathogens, tolerance to acid and bile, and our prior demonstration in a study conducted in 80 human volunteers that *L salivarius* UCC4331, delivered in either a milk drink or a yogurt, is well tolerated, successfully colonizes the gastrointestinal tract, and produces expected quantitative and qualitative changes in the gut flora.[59–61] Furthermore, the introduced lactobacilli were found to stimulate a mucosal but not a systemic immune response. Finally, related strains had been shown to be of benefit in the prevention of human diarrheal conditions such as toddlers' diarrhea and *Clostridium difficile*–related, antibiotic-associated diarrhea.[35,36,69–74]

B infantis 35624 was chosen from among several bifidobacterium probiotic strains developed in University College Cork based on tolerance to acid and bile and the demonstration in mouse models of inflammatory bowel disease that *B infantis* 35624 may prove highly effective in beneficially altering gut flora and in alleviating the inflammatory changes that characterize these models.[38,75]

What is the mechanism of action of this probiotic in IBS? This organism has been shown to exert potent immune effects, including the promotion of anti-inflammatory and the inhibition of proinflammatory cytokines. For example, oral administration of the bifidobacterium used in this study exerted a profound anti-inflammatory effect in the IL-10 knockout mouse, a potent model of inflammatory bowel disease that was associated with a suppression of the proinflammatory cytokines interferon gamma, tumor necrosis factor α, and IL-12 while preserving activity of the anti-inflammatory cytokine transforming growth factor β.[38] This is of particular interest given recent reports of low-grade inflammation and a similar pattern of cytokine activation among patients with IBS.[23–27] Our finding in this study of a cytokine ratio in IBS skewed toward a Th1, proinflammatory profile provides further support for this hypothesis. Based on these and other observations,[26] it is tempting to speculate that failure to adequately down-regulate a proinflammatory response following a precipitating event (eg, gastrointestinal infection) may sustain the IBS state. This study has taken this concept one step further; by demonstrating a normalization of the IL-10/IL-12 ratio in the bifidobacteria-fed subjects alone, and in parallel with symptomatic improvement, we provide the first evidence for efficacy for an anti-inflammatory approach in IBS.

While this study protocol did not include biopsies, thus precluding an evaluation of the effects of this therapy on colorectal mucosal histology or immune activation, others have recently shown potent anti-inflammatory effects at the mucosal level for probiotic preparations that contained bifidobacteria.[76,77] In this context, we must also acknowledge that the mucosa is functionally and operationally distinct from the systemic or peripheral immune systems and that direct relationships between these compartments have not been shown in humans. We are currently exploring these relationships in humans in IBS and in response to probiotic therapy; meanwhile, we would draw attention to studies from our group, albeit in a murine model, showing parallel mucosal and systemic cytokine responses to the same probiotic strains as used in this study.[38] The hypothesis that bifidobacterium is acting through an anti-inflammatory mechanism is indeed an attractive one; this effect could abrogate the induction of hypersensitivity, hyperalgesia, altered central perception, and dysmotility by inflammatory triggers.[78] There is indeed some preliminary evidence that probiotic administration may diminish visceral hypersensitivity in animal models.[79,80] Furthermore, effects on motility and perception could also go some way toward explaining the beneficial effects on bloating, given current concepts on the roles of altered gas transit and visceral hypersensitivity in the pathogenesis of this symptom.[81–83]

Of the other putative effects of probiotics, an effect on stool bulking would seem unlikely because we failed to observe any change in either stool consistency or frequency. This apparent independence of the effects of

bifidobacterium from any change in stool frequency or form has important clinical implications, implying that this therapeutic approach may be applicable to all patients with IBS, irrespective of stool pattern. Whether qualitative or quantitative changes in small intestinal or colonic flora accompany probiotic feeding and thereby alter flora-mucosal interactions to the benefit of the host cannot be determined from this study. We do know from the stool recovery studies that the organisms survived transit through the intestine, but we did not assess in this study interactions between the indigenous flora and the administered probiotics. These same studies from our group have shown continued recovery of orally administered probiotic organisms from stool for 3 or more weeks in 12.5% of healthy volunteers,[61] an observation that may explain the persistence of benefit from bifidobacterium in this study into the washout phase. Furthermore, others[84,85] have shown that probiotic organisms can adhere to the colonic mucosa and can continue to be recovered from colonic biopsy specimens long after the discontinuation of oral feeding and after they cease to be recovered from fecal samples.

Whether IBS is accompanied by quantitative or qualitative changes in the bacterial flora of the small or large intestine remains a contentious issue; while some have described bacterial overgrowth in the small intestine[28–30] and qualitative alterations in the fecal flora[39,40,47] and increased bacterial fermentation[41] in IBS, others have failed to replicate these findings.[12,31,81] A reduction in bacterial fermentation by a modulation of the composition of the flora could certainly contribute to the alleviation of the "gas-related" symptoms that are so common in IBS[82] and that seem to reflect a selective defect in intestinal gas transport.[83] Probiotics have also been shown to modulate enteroendocrine cell populations in the mouse intestine.[86]

Other studies have evaluated the response of IBS to a number of probiotic preparations. In a recent review, Hamilton-Miller, while drawing attention to the shortcomings of prior trials in terms of study design, concluded that there was, overall, sufficient evidence of efficacy to warrant further evaluation.[55] Most studies reviewed were small in size and almost certainly underpowered to show anything other than a very striking benefit. Several did not verify bacterial transit and survival by confirmatory stool studies. Many different organisms and strains were used, and dosages varied from as little as 10^5 to 10^{13}. Furthermore, some, including a recent study, used probiotic "cocktails" rather than single isolates, rendering it impossible to induce what, if any, were the active moieties.[53] Nevertheless, some positive results were noted. Niedzielin et al reported resolution of abdominal pain in all 20 patients treated for 4 weeks with *Lactobacillus plantarum* 299V, in contrast to only 11 of 20 patients who received a placebo,[48] and Halpern et al noted a significant reduction in an IBS symptom index with a capsule containing 5×10^9 heat-killed *Lactobacillus acidophilus*.[44] O'Sullivan and O'Morain, while failing to detect an effect of *Lactobacillus casei* GG on overall symptomatology, did note a trend toward reduction in bloating.[46] Nobaek et al, using *L plantarum* (DSM 9843),[45] described a similar benefit in terms of relief of bloating, as did Kim et al in their evaluation of the probiotic "cocktail" VSL#3.[53]

In contrast, the study reported herein provides, for the first time, clear evidence for a benefit in IBS for a clearly-defined single-organism probiotic preparation and thereby suggests that some strains, and bifidobacterium in particular, may be more effective that others for this indication. Furthermore, this symptomatic response was associated with a normalization of the ratio of an anti-inflammatory to a proinflammatory cytokine, suggesting an immune-modulating role for this organism, in this disorder. While the limitations inherent to the study mandate that its findings be interpreted with caution, it should at the very least prompt large randomized controlled trials of this bifidobacterium strain in IBS as well as detailed explorations of its mechanism(s) of action.

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Wirsung of the Duct of Wirsung

Johann Georg Wirsung (1600–1643) was born in Augsburg, Bavaria. From there he emigrated to the famed school of medicine at Padua where he became a prosector in anatomy. It was there that one of his students Maurice Hoffman came across a pancreatic duct in a rooster and displayed the dissection to his instructor. Thereupon, Wirsung set out to demonstrate a counterpart in a human cadaver. This accomplished, Wirsung communicated his finding, together with a detailed illustration, in a letter to Jean Riolan (1577–1657), a senior colleague in Paris. In the following year 1643, Wirsung was fatally felled by a gunshot fired by an unknown assassin, presumably the culmination of a dispute over priority of the discovery. The lesson, if any, is perhaps to refrain from pressing one's claim to originality with undue vigor.

—Contributed by WILLIAM S. HAUBRICH, M.D.

The Scripps Clinic, La Jolla, California

Bergey's Manual of Systemic Bacteriology Scardovi

see page 1424

illen ends. After prolonged incubation, they usually fragment into
ohtheroidal or coccoid elements of varied size and shape.
The cell wall contains glucosamine, muramic acid, alanine, glutamic
d, lysine, ornithine, and aspartic acid. Rhamnose is the predominant
l wall sugar, but glucose and fucose may be present in trace amounts.
Microcolonies on agar media and initial growth in liquid media are
ially filamentous. Mature colonies are small, opaque, smooth, entire,
ivex, with a dark central region. Rough colony variants occur occa-
ially. Pigmentation is not evident. In liquid media, growth is gran-
r or flocculent forming a white sediment without turbidity.
The optimum growth temperature is approximately 30°C; poor or no
wth at 37°C.
The organism does not grow on media lacking organic nitrogen. In
ition, little if any growth is obtained in certain chemically defined
lia or media containing simple peptones.

Other descriptive and differential characteristics are listed in Tables 15.47 and 15.49.

Using the fluorescent antibody technique, no cross-reactivity was observed between "*A. humiferus*" and other *Actinomyces* or *Rothia* species. A slight cross-staining obtained with *Corynebacterium (Bacterionema) matruchotii* antiserum was considered nonspecific.

The natural habitat of "*A. humiferus*" appears to be organically rich soil from which the organism may be recovered in high numbers. Experimental infection could not be induced in mice after intraperitoneal injection of washed saline cell suspensions.

The mol% G + C of the DNA is 73 on average (density gradient).

Type strain: ATCC 25174.

Genus Bifidobacterium *Orla-Jensen 1924, 472*[AL]

VITTORIO SCARDOVI

Bi.fi.do.bac.te'ri.um. L. adj. *bifidus* cleft, divided; Gr. dim. n. *bakterion* a small rod; M.L. neut. n. *Bifidobacterium* a cleft rodlet.

od[illegible] of various shapes: short, regular, thin cells with pointed
, coccoidal regular cells, long cells with slight bends or protuber-
s or with a large variety of branchings; pointed, slightly bifurcated
-shaped or spatulated extremities; single or in chains of many
ents; in star-like aggregates or disposed in "V" or "palisade"
igements. Colonies smooth, convex, entire edges, cream to white,
ming and of soft consistency. Gram-positive, non-acid-fast;
pore-forming, nonmotile. Cells often stain irregularly with
ylene blue. Anaerobic; some species can tolerate O_2 only in the
nce of CO_2. Optimum growth temperature 37–41°C; minimum
h temperatures 25–28°C maximum 43–45°C. Optimum pH for
l growth 6.5–7.0; no growth at 4.5—5.0 or 8.0–8.5
charoclastic. Acetic and lactic acid are formed primarily
e molar ratio of 3:2. CO_2 is not produced (except in the
dation of gluconate). Small amounts of formic acid, ethanol and
ic acid are produced. Butyric and propionic acid are not
iced. Glucose is degraded exclusively and characteristi-
by the fructose-6-phosphate shunt in which fructose-6-phos-
tolase (F6PPK-EC 4.1.2.22) cleaves fructose-6-phosphate into
phosphate and erythrose-4-phosphate. End products are formed
h f[illegible] sequential action of transaldolase (EC 2.2.1.2), transketo-
EC [illegible].1.1), xylulose-5-phosphate phosphoketolase (EC 4.1.2.9)
z[illegible] of EMP acting on glyceraldehyde-3-phosphate. Additional
ani[illegible]ormic acid may be formed through a cleavage of pyruvate.
ose-6-phosphate dehydrogenase (EC 1.1.1.49, $NADP^+$- or
dependent) generally not determinable.
lase-negative except that *B. indicum* and *B. asteroides* are cat-
positive when grown in the presence of air with or without added

onium is generally utilized as a source of nitrogen.
+ C content of DNA (Bd or T_m) varies from 55–67 mol%.
rganisms occur in the intestine of man, various animals and
ees; found also in sewage and human clinical material.
species: *Bifidobacterium bifidum* (Tissier) Orla-Jensen 1924,

Further Descriptive Information

ogy

llular morphology and its variations, as affected by different
onditions, have been widely investigated (see Poupard, Husain
is, 1973, for references). However, recent discoveries of new
om a variety of habitats have permitted a clearer picture of
ology of the genus.
ison of the cell morphology of large numbers of strains grown anaerobically (GasPak system, BBL) in stabs of trypticase-phytone-yeast extract medium (TPY) showed that some species had distinct cell shapes or arrangements which might be of help in their recognition; these traits are reported in Figs. 15.96–15.98.

Outstanding are the well known amphora-like cells of *B. bifidum* (Sundman et al., 1959) (Fig. 15.96*A*), the V or palisade arrangement of cells in *B. angulatum* (Fig. 15.96*D*), the linear groups of globular elements in *B. catenulatum* (Fig. 15.96*E*), the long chains of regular cells in *B. pullorum* (Fig. 15.97*A*), the middle-enlarged cells of *B. animalis* (Fig. 15.97*B*), the large cellular dimensions in *B. magnum* (Fig. 15.97*D*), the small cells of *B. minimum* (Fig. 15.97*F*), and the unusual starlike arrangements of cells in *B. asteroides* (Fig. 15.98*A*). The cellular shape most frequently encountered in those species not having distinct morphology (see Table 15.50) as observed in TPY stabs (see above), is depicted in Fig. 15.98*D*. Details are given under single species description.

B. asteroides (starlike clusters) and *B. indicum* (small rods or coccobacilli), the species with the most nonbifid-like morphology in the classic sense, show features common to the morphology of the other bifids only when grown in nutritionally deficient media (Scardovi and Trovatelli, 1969), which seems to be a general trend in this group of bacteria (Sundman and Björksten, 1958; Glick et al., 1960).

Cell Wall Structure

The most extensive study of cell wall murein structure of bifidobacteria has been made by O. Kandler and collaborators (reported later in Table 15.50). Closely related species can be clearly distinguished on this basis, i.e. *B. boum* from *B. thermophilum* or *B. minimum* from *B. subtile*.

On the basis of murein structure, bifidobacteria are more closely related to *Lactobacillaceae* than to *Actinomycetaceae* (Kandler and Lauer, 1974).

Lipid Cellular Composition

Some species of *Bifidobacterium* and *Lactobacillus* were studied by Exterkate et al. (1971): differences in polyglycerol phospholipids and aminoacyl phosphatidylglycerol were found to be of help in differentiating the two genera. The effects of growth conditions on the lipid and ionic composition of *B. bifidum* subsp. *pennsylvanicum* have been recently studied by Veerkamp (1977a, b).

Ultrastructure

The ultrastructure of bifidobacteria has received little attention. Overman and Pine (1963) first reported ultrastructure micrographs of *B. bifidum* subsp. *pennsylvanicum*. Recently, Zani and Severi (1982)

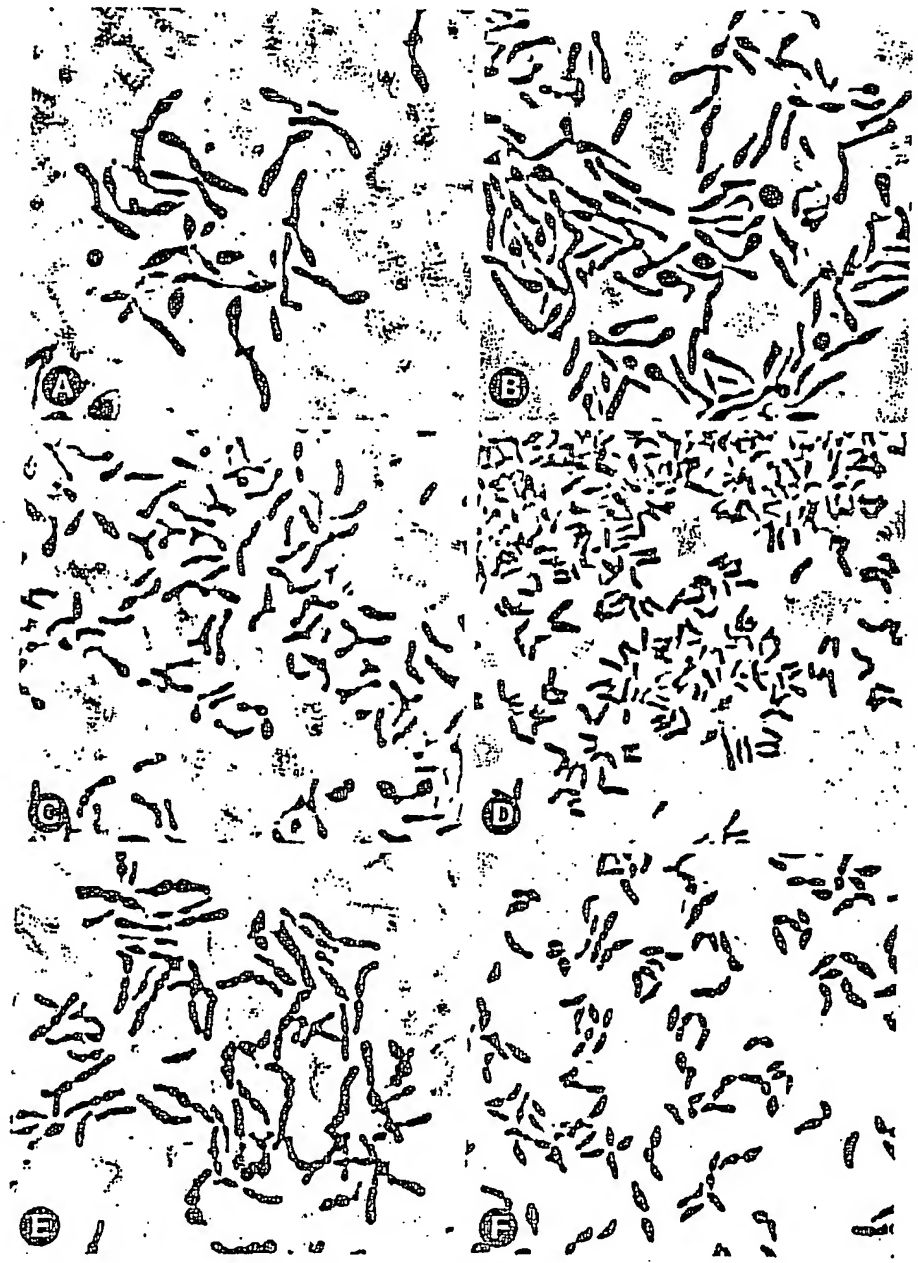


Figure 15.96. Cellular morphology in the genus *Bifidobacterium*. Cells grown in TPY agar stabs. *A, B. bifidum; B, B. longum; C, B. breve; D, B. angulatum*; *E, B. catenulatum*; and *F, B. globosum*. Phase-contrast photomicrographs, × 1500.

made a more extensive investigation of the ultrastructure of *B. bifidum* strain S28a of Reuter (ATCC 15696). Comparative studies on the ultrastructure of species comprising the genus have not yet been made.

Nutrition

Since its first isolation from human infants' feces (György, 1953) and its designation as *Lactobacillus bifidus* var. *pennsylvanicus* (György and Rose, 1955), this organism, the growth of which is stimulated by human milk, has been the object of numerous nutritional studies designed either to elucidate the properties of the bifidus factor(s) present in human milk, or to find a substitute for it (see reviews by Poupard et al., 1973; Yoshioka et al., 1968; Nakamura and Tamura, 1972; Nichols et al., 1974; György et al., 1974; Yazawa et al., 1978; Beerens et al., 1980).

However, as only the György strain and a few others of ill defined taxonomy were used for these studies, they have little bearing on our knowledge of the nutritional requirements of the genus, particularly now that so many new species are known. The growth factor requirements of twelve species are reported later in Table 15.53; they form a very heterogeneous group and the vitamin requirements seem unrelated to the ecological distribution of the species.

Bifidobacteria are able to utilize ammonium salts as sole source of

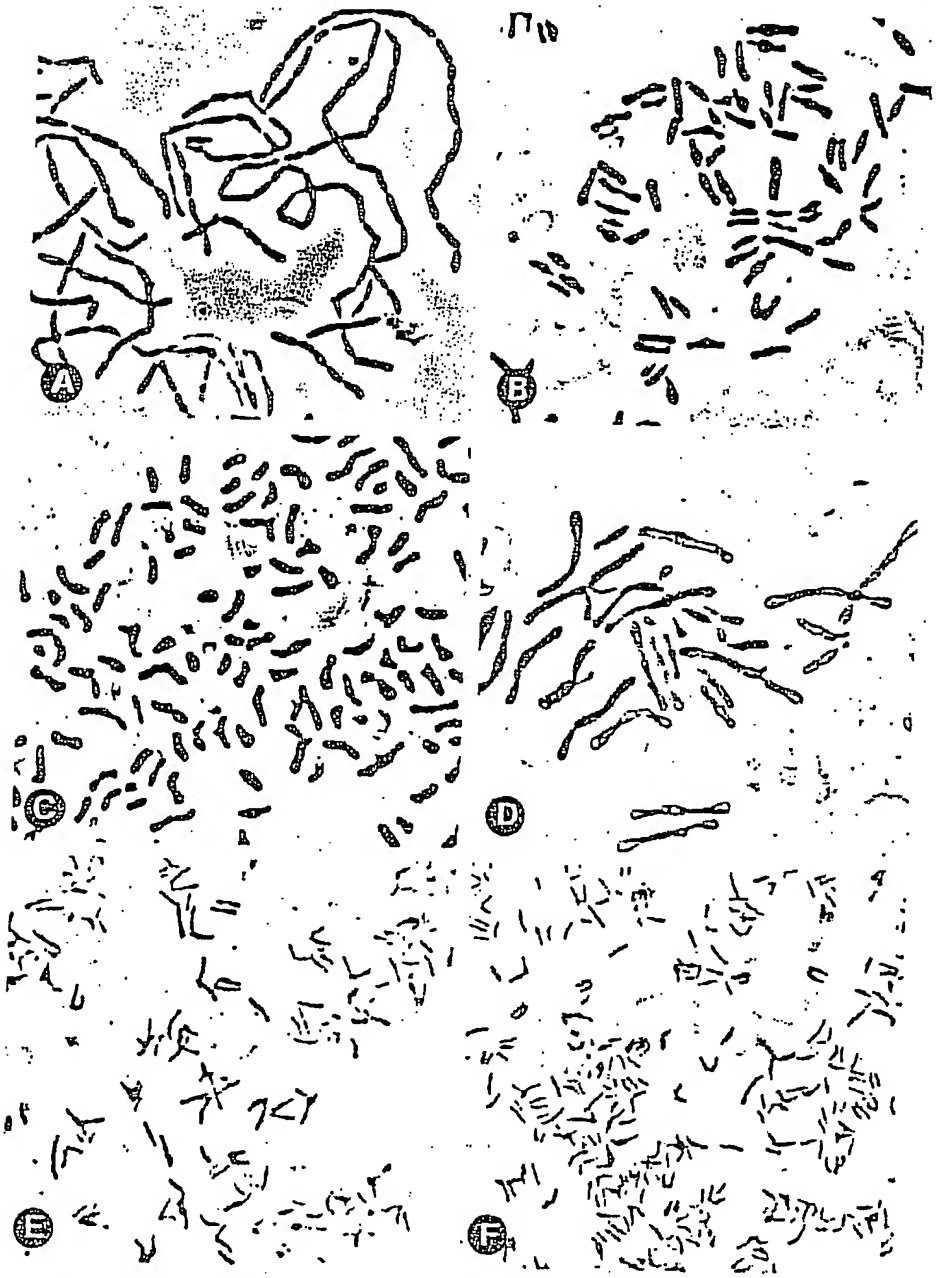


Figure 15.97. Cellular morphology in the genus *Bifidobacterium*. Cells grown in TPY agar stabs. *A, B. pullorum; B, B. animalis; C, B. cuniculi; D, B. magnum; E, B. subtile;* and *F, B. minimum*. Phase-contrast photomicrographs, × 1500.

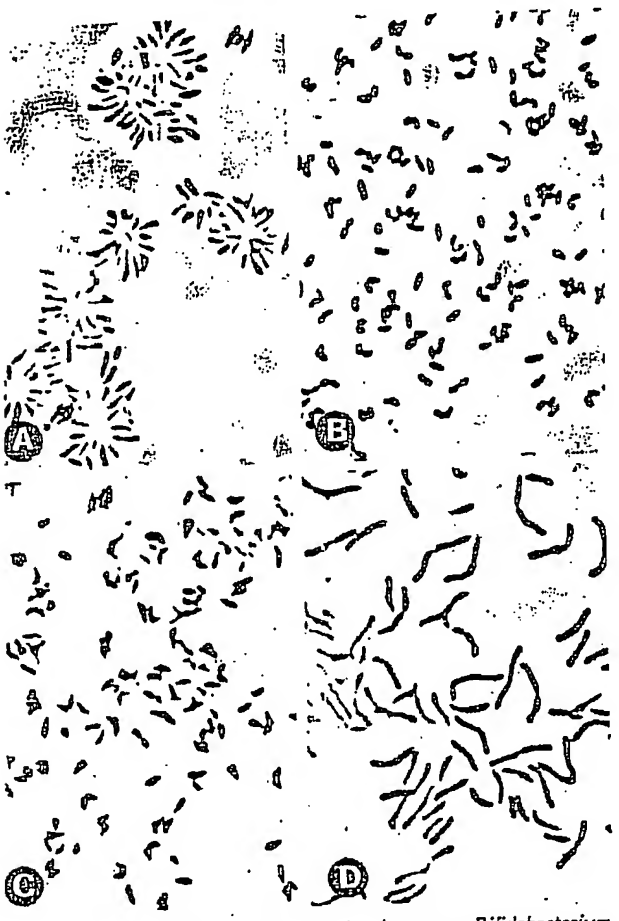
igen. This finding, reported first by Hassinen et al. (1951), is valid ıost species of the genus, but *B. suis*, *B. magnum*, *B. choerinum* *B. cuniculi* will not grow without organic nitrogen (Matteuzzi et 978). (*B. choerinum* is reported under the provisory name of "*B. ıense*" in this paper). The species which grow without organic gen excrete considerable amounts of various amino acids into the um: e.g. *B. bifidum* can produce up to 150 mg/liter threonine. r active amino acid producers are *B. thermophilum*, *B. adolescentis*, *ntium*, *B. animalis* and *B. infantis*. The amino acids generally ıced in the largest amounts are alanine, valine and aspartic acid :euzzi et al., 1978).

Analog-resistant mutants were obtained from *B. thermophilum* (*B. ruminale*) showing increased production of isoleucine and valine (Matteuzzi et al., 1976; Crociani et al., 1977).

Carbohydrate Metabolism

The fermentation of hexose occurs in the genus *Bifidobacterium* through the following sequence of reactions (bifid shunt) (Scardovi and Trovatelli, 1965; De Vries et al., 1967, Veerkamp, 1969b).

$$\text{Fructose-6-P} + \text{iP} \xrightarrow{\text{F6PPK}} \text{erythrose-4-P} + \text{acetyl-P} + H_2O$$



igure 15.98. Cellular morphology in the genus *Bifidobacterium*. ells grown in TPY agar stabs. *A, B. asteroides; B, B. indicum; C, B. ıryneforme; and D, B. infantis*. Phase-contrast photomicrographs, × 500.

$$\text{Erythrose-4-P} + \text{fructose-6-P} \xrightarrow{\textit{transaldolase}} \text{sedoheptulose-7-P} + \text{glyceraldehyde-3-P}$$

$$\text{Sedoheptulose-7-P} + \text{glyceraldehyde-3-P} \xrightarrow{\textit{transketolase}} \text{ribose-5-P} + \text{xylulose-5-P}$$

$$\text{Ribose-5-P} \xrightarrow{\textit{isomerase}} \text{xylulose-5-P}$$

$$2\ \text{xylulose-5-P} + \text{iP} \xrightarrow{\textit{xylulose phosphoketolase}} 2\ \text{glyceraldehyde-3-P} + 2\ \text{acetyl-P} + 2\text{H}_2\text{O}$$

$$3\ \text{acetyl-P} \rightarrow 3\ \text{acetate}$$

$$2\ \text{glyceraldehyde-3-P} \xrightarrow{\textit{EMP enzymes}} + 2\ \text{lactate}$$

$$2\ \text{hexose} \rightarrow 3\ \text{acetate} + 2\ \text{lactate}$$

However, the theoretical ratio of acetate 1.5:lactate 1.0 is scarcely ver found in growing cultures of bifidobacteria: phosphoroclastic cleav- ge of some pyruvate to formic acid and acetyl phosphate and reduction f acetyl phosphate to ethanol can often alter the fermentation balance n favor of the production of acetate and some formic acid and ethanol. iven X as the amount of formic acid produced, the general reaction postulated was: glucose → (1.5 + 0.5 X) acetate + (1.0 − X) lactate + 0.5 X ethanol + X formate (De Vries and Stouthamer, 1968, Lauer and Kandler, 1978).

Glucose-6-phosphate dehydrogenase and aldolase are claimed to be absent or not detectable, thus ruling out the monophosphate pathway and the glycolytic system (De Vries and Stouthamer, 1967). However, low but detectable levels of these two enzymes were found in some species (Scardovi and Sgorbati, 1974; see under description of species).

The enzymes of the Leloir pathway of galactose metabolism, i.e. galactokinase (EC 2.7.1.6), hexose-1-phosphate uridylyltransferase (EC 2.7.7.12) and UDP galactose 4-epimerase (EC 5.1.3.2) are constitutive in glucose-grown cells of *Bifidobacterium*, whereas in other microorganisms these enzymes are induced by galactose or fucose (Lee et al., 1980).

The existence of UPD galactose pyrophosphorylase (Lee et al., 1978) suggests that, at least in species of the genus from human sources, an alternative pathway of galactose is operative (Lee et al. 1979).

The enzymatic carboxylation of phosphoenolpyruvate to oxaloacetate in some bifidobacteria from human feces and from the honey bee, has been compared with the corresponding activity in strains of *Actinomyces bovis* and *Actinomyces israelii*: in bifids this activity is independent of the phosphate acceptor and is irreversible, whereas in *Actinomyces*, it is inosine or guanosine diphosphate-dependent (Chiappini, 1966).

Urease Activity

Four hundred and fourteen strains representing 21 species of the genus were surveyed for their urease activity. The strongest ureolytic strains belong mostly to *B. suis*, in which more than 80% of the strains studied are ureolytic. Ureolytic strains were found in all species except for *B. cuniculi*. The enzyme is apparently not inducible: urea and organic nitrogen do not influence urease production. In *B. breve* and *B. longum*, i.e. "human" bifid species, less than 10% of strains are ureolytic and *B. bifidum* is only weakly ureolytic (Crociani and Matteuzzi, 1982).

Anaerobiosis

Bifidobacteria are anaerobic microorganisms: they do not develop in plates under aerobic conditions. However, the sensitivity to oxygen is different among different strains and species, and its intimate reasons are equivocal (De Vries and Stouthamer, 1969). While most species do not develop in slants incubated under an atmosphere of CO_2-enriched air (air 90%; CO_2 10%) *B. globosum*, *B. thermophilum* and *B. suis* do so without the cells becoming catalase-positive even if hemin is added to the medium; *B. asteroides* grows under these conditions and becomes catalase-positive; *B. indicum* behaves similarly, but is catalase-positive only if hemin is added to the medium (Scardovi et al., 1969; Scardovi and Trovatelli, 1969; Matteuzzi et al., 1971).

Nitrate Reduction

Bifidobacteria are generally claimed not to reduce nitrate. However, cells grown in the presence of lysed red cells may be capable of nitrate reduction. Cytochrome *b* and cytochrome *d* are synthesized under these conditions of growth (van der Wiel-Korstanje and De Vries, 1973).

Enzymes Used for Species or Group Differentiation

Fructose-6-phosphate phosphoketolase is the characteristic key enzyme of the "*bifid shunt*." Tested with fructose-6-phosphate as substrate (see below), it is apparently absent in anaerobic Gram-positive bacteria of "pseudobifid" morphology, i.e. *Arthrobacter*, *Propionibacterium*, *Corynebacterium* and *Actinomyces* (Scardovi and Trovatelli, 1965).

Starch gel electrophoresis revealed three types of F6PPK in bifido-bacteria (see Table 15.50) (Scardovi et al., 1971a): the most anodal was detected in species found in the intestine of honey bees; the type found in species from humans (*human* type) migrated less and the least anodal type characterized the species found in animals (*animal*

type). F6PPK from *B. globosum* (*animal* type) and *B. dentium* (*human* type) have been purified (Sgorbati et al., 1976). The *animal* type has properties similar to that found in *Acetobacter xylinum* (Schramm et al., 1958), and is also active toward xylulose-5-P; the *human* type has different properties (activators, pH range of activity, heat inactivation) and cleaves only fructose-6-P (Sgorbati et al., 1976).

These ecological groups of *Bifidobacterium* species were distinguished also on immunological basis (Sgorbati and London, 1982, see below). Isozyme patterns could also be used to identify species. Isozymes of transaldolase and 6-phosphogluconate dehydrogenase (6PGD in Table 15.50) were studied by starch gel electrophoresis in 1206 strains belonging to each of the 24 species (Scardovi et al., 1979a). Fourteen isozymes of transaldolase and 19 of 6PGD were identified and numbered: patterns or zymograms were obtained for each species (see Table 15.50 for relavant data): 60% of the strains could be identified on that basis. An additional 20% of the strains were assigned to species on the basis of the electrophoretic behavior of their 3-phosphoglyceraldehyde dehydrogenase (Scardovi et al., 1979a).

Antisera against eight purified transaldolases further established natural relationships among the species (Sgorbati, 1979; Sgorbati and Scardovi, 1979; Sgorbati and London, 1982). Transaldolases selected on the basis of their electrophoretic behavior (see above), were from *B. infantis*, *B. angulatum*, *B. globosum*, *B. thermophilum*, *B. suis*, *B. cuniculi*, *B. minimum* and *B. asteroides*. On the basis of microcomplement fixation data the indices of dissimilarity and the immunological distances were determined; the results, in terms of taxonomic distances, are shown diagrammatically in Fig. 15.99.

The segregation of the species into four distinct clusters which coincide so neatly with the groups which can be made on the basis of their ecological distribution, suggests that a "subdivision of the genus into four subgenera would more accurately reflect the group's natural history" (Sgorbati and London, 1982).

The various purified transaldolases cannot be distinguished on the basis of the usual parameters such as molecular weight, substrate affinity, pH range, acceptors, etc. (Scardovi, unpublished).

Plasmids

When 1461 isolates representing the 24 species of the genus were examined for the presence of plasmids, ~20% of them were found to contain plasmids. However, only four species have these elements, namely *B. longum*, the predominant bifid species in the human intestine, *B. globosum*, the most common bifid in animals, and *B. asteroides* and *B. indicum*, species found exclusively in the intestine of honey bees. *B. longum* strains have multiple-plasmid patterns (1.25–9.5 MDa); *B. globosum* strains contain one plasmid each of three classes of molecular weight (13.5, 24.5 and 46 MDa); multiple patterns were seen in *B. asteroides* (1.2–22 MDa); 60% of the plasmid-bearing *B. indicum* isolates contained one 22 MDa plasmid (Sgorbati et al., 1982). It is noteworthy that *B. infantis*, the species most closely related to *B. longum*, does not contain plasmids, although strains of both species were isolated from the same specimens. No phenotypic properties have been correlated as yet with the plasmids (Sgorbati et al., 1982).

The thirteen plasmid patterns of *B. longum* contain a few homologous structures only, whereas the fourteen patterns found in *B. asteroides* are structurally more heterogeneous (Sgorbati, unpublished).

Strains of *B. longum* liberate phage particles after UV or mitomycin c treatment, but there is no correlation with their plasmid complement (Sgorbati, unpublished).

Resistance to Antibiotics

Resistance (with respect to achievable serum levels) to kanamycin, neomycin, streptomycin, polymyxin, gentamicin, nalidixic acid and metronidazole was a general feature among 15 *Bifidobacterium* species studied (including strains from human habitats and those from honey bees). Oleandomycin, lincomycin, clindamycin, vancomycin, penicillin G, ampicillin, erythromycin, bacitracin, chloramphenicol and nitrofurantoin were strongly inhibitory; sensitivity to tetracycline was inter- and intraspecifically variable (Matteuzzi, unpublished). These results extend and substantially confirm those already reported concerning mainly *B. bifidum*, *B. longum* and *B. adolescentis* (Miller and Finegold, 1967).

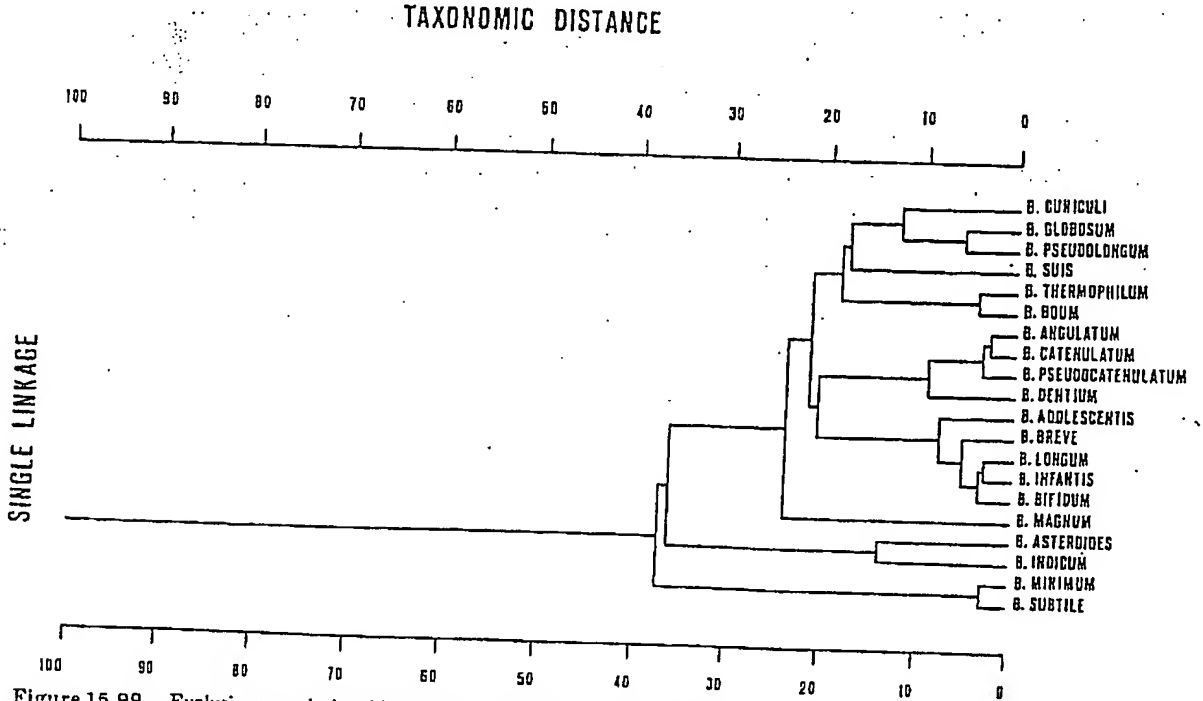


Figure 15.99. Evolutionary relationships among the bifidobacteria. The data were obtained with eight antitransaldolase sera (from Sgorbati, unpublished).

The genus *Bifidobacterium*, therefore, exhibits uniform sensitivity ward the most common antibiotics; however, sensitivity toward an- biotics such as polymyxin, neomycin, kanamycin, streptomycin, gen- micin or nalidixic acid varies greatly within species, ranging from)–500 or more μg/ml antibiotic.

The electrophoretic patterns of cellular proteins (polyacrylamide gel . ctrophoresis (PAGE)) procedure of Moore et al., 1980) was used to .nfirm the taxonomic identification of 1094 bifidobacteria strains elonging to all known species of the genus. Excellent correlation was ound between electrophoretic data and the classification of the genus resented here: on this basis two species were revived (*B. coryneforme* nd *B. globosum*) and two others were proposed for previously recog- nized DNA homology groups (*B. minimum* and *B. subtile*) (Biavati et al., 1982).

This procedure was confirmed as one of the most useful in identifying unknown bifid isolates. In addition, a sort of "*genus band*" is clearly visible in all bifid gel electrophoresis patterns where the band migrates in the same position, with the exception of *B. boum* where it is somewhat less anodic. This appears to be a useful indication that an unknown organism belongs to the genus (Biavati et al., 1982).

Possible Pathogenicity

Most strains of *B. dentium* have been isolated from human dental caries, this species being the only bifid found in this site. (Scardovi and Crociani, 1974). Strains of *B. appendicitis* (a synonym of *B. dentium* Scardovi and Crociani, 1974) were found by Prévot in human clinical material (Prévot et al., 1967). *Actinomyces eriksonii* Georg, Robertstad, Brinkman and Hicklin, 1965, (named later *Bifidobacterium eriksonii* by Holdeman and Moore, 1970), recognized genetically as *Bifidobacterium dentium* (Scardovi et al., 1979a), was isolated primarily from human abscesses. Most of the strains studied with PAGE procedure by Biavati et al. (1982), were isolated from a variety of human clinical material and from human dental plaque. *B. longum* and *B. breve* occur occasionally in human clinical material (Biavati et al., 1982). Thus, *B. dentium* appears to have the most possible pathogenic potentiality.

Isolation Procedures

A large variety of media have been devised for isolating or enumerating bifidobacteria in natural habitats. Ingredients of substrates have been tomato juice, sheep or horse blood, human milk, liver or meat extracts, a variety of peptones etc. (for references, see Scardovi, 1981).

In order to improve selectivity, antibiotics or other ingredients have been used: kanamycin (Flnegold et al., 1971); neomycin (Mata et al., 1969); paramomycin, neomycin, sodium propionate and lithium chloride (Mitsuoka et al., 1965); sorbic acid or sodium azide (Haenel and Müller-Beuthow, 1956; Haenel and Müller-Beuthow, 1957). The known bifidobacteria are resistant to certain antibiotics (see above) but the intraspecific variations are so large that the use of such antibiotics would make isolation unreliable. At present, the preference should be given to substrates that permit satisfactory growth of the largest number of bifidobacteria types presently known. The ingredients of choice are trypticase and phytone (BBL) and the formula which in our hands proved to be satisfactory for isolating the bifids from all known habitats (TPY medium) is: trypticase (BBL), 10 g; phytone (BBL), 5 g; glucose, 5 g; yeast extract (Difco), 2.5 g; Tween 80, 1 ml; cysteine hydrochloride, 0.5 g; K_2HPO_4, 2 g; $MgCl_2 \cdot 6\,H_2O$, 0.5 g; $ZnSO_4 \cdot 7\,H_2O$, 0.25 g; $CaCl_2$, 0.15 g; $FeCl_3$, a trace; agar, 15 g; distilled water to 1000 ml. Final pH is about 6.5 after autoclaving at 121°C for 25 min; dilutions can be made with the same liquid medium.

Petri dishes with vents are incubated at 39–40°C in anaerobic jars with palladium catalyst, filled with a gas mixture of 10% CO_2 and 90% H_2 (or GasPak system). Colonies are transferred to stabs of the same medium with 0.5% agar. After growth has recurred, stabs are kept at 3–4°C in the anaerobic jar. Transfers should be made every 2 weeks.

Procedure for Testing Special Identification Characters

Identification of a bacterial strain as *Bifidobacterium* is unreliable unless special procedures are used. Morphology can be misleading on account of unusual and unique traits shown by some species (*B. angulatum*, *B. asteroides*, *B. pullorum* etc.). Cultural and physiological characters are largely shared by other bacteria such as *Actinomyces*, *Corynebacterium* and *Lactobacillus*. Identification of the fermentation products by gas chromatography (Holdeman et al., 1977) may be difficult, especially for inexperienced workers, as side reactions forming substantial and variable amounts of formic and succinic acids and ethanol may occur.

The most direct and reliable characteristic assigning an organism to the genus *Bifidobacterium* is that based on the demonstration of F6PPK in cellular extracts. Proofs of the validity of this test have been furnished by (a) isolation from the bovine rumen and from sewage of anaerobic bacteria with the morphology and gross physiology of bifidobacteria but which do not possess F6PPK (Scardovi, unpublished) and (b) recognition as bifids on this basis, of bacteria of nonbifid morphology such as *B. pullorum* (Trovatelli et al., 1974) and bifids from honey bees (Scardovi and Trovatelli, 1969).

Fructose-6-Phosphate Phosphoketolase (F6PPK) Test

Reagents: 1) 0.05 M phosphate buffer pH 6.5 plus cysteine 500 mg/liter; 2) a solution containing NaF, 6 mg/ml, and K or Na iodoacetate, 10 mg/ml; 3) hydroxylamine HCl, 13.9 g/100 ml of water, freshly neutralized with NaOH to pH 6.5; 4) trichloroacetic acid (TCA), 15% (w/v) in water; 5) 4 M HCl; 6) $FeCl_3 \cdot 6H_2O$ 5% (w/v) in 0.1 M HCl; 7) fructose-6-phosphate (Na salt: 70% purity), 80 mg/ml in water.

The formation of acetyl phosphate from fructose-6-phosphate is evidenced by the reddish violet color (absorption maximum at 505 nm) formed by the ferric chelate of its hydroxamate (Lipmann and Tuttle, 1945).

Procedure

Cells harvested from 10 ml TPY broth are washed twice with buffer 1) and resuspended in 1.0 ml of the same buffer. The cells are disrupted by sonication in the cold, and 0.25 ml each of reagents 2) and 7) are added to the sonicate. After 30 min incubation at 37°C, the reaction is stopped with 1.5 ml of reagent 3). After 10 min at room temperature, 1.0 ml each of reagents 4) and 5) are added. The mixture may be left at room temperature before the final addition of 1.0 ml of the color-developing reagent 6). Invert tube for mixing. Any reddish violet color that develops immediately is taken as a positive result. A tube without fructose-6-P can serve as blank to aid the visual comparison; the color becomes more evident after standing, which allows particles to settle. Warning: avoid heating during sonication because of heat sensitivity of the enzyme.

Electrophoresis methods to detect enzymes. The starch-gel horizontal electrophoresis system of Smithies (1955) is recommended.

Transaldolase. Tris(hydroxymethyl)aminomethane (Tris) 16.3 g and citric acid monohydrate 9.0 g/liter (pH 7.0) is used as bridge buffer; dilute this 1:15 and use as gel buffer.

Hydrolyzed starch, 90 g, (Connaught Laboratories Ltd., Willondale, Ontario, Canada) is added per liter of buffer; the mixture is boiled for 5 min (keep agitated) and gas removed under reduced pressure. The liquid is poured into a plastic three-frame mold (12.0 × 37.0 × 0.9 cm are suitable to accommodate 12 samples at a time).

Samples of bacterial extracts (cells suspended in 0.05 M phosphate buffer pH 7.0, sonicated and centrifuged), 5–10 μl in 0.5 × 0.5 cm Whatman 3-mm paper cuts, are generally run for 15–20 h with a current of 15–20 mA.

The middle slab is used preferably for staining by the flooding technique. The developing solution contains (per 100 ml of distilled water): fructose-6-P (Na salt, 98% purity, Sigma), 400 mg; sodium arsenate, 370 mg; glycine, 240 mg; NAD, 13 mg; D-erythrose-4-phosphate (60–75% purity, Sigma), 16 mg; phenazine methosulfate, 2 mg; nitro blue tetrazolium (NBT, Sigma), 20 mg; and about 130–150 IU of glyceraldehyde-3-phosphate dehydrogenase.

6-Phosphogluconate dehydrogenase (6 PGD). Use trisodium citrate-2 H_2O 120 g/liter (pH 7.0 with citric acid) as bridge buffer; histidine 0.75 g plus NaCl 1.5 g/liter (pH 7.0) as gel buffer. Prepare the gel as

for detecting transaldolase. The developing solution is made as follows: 0.5 M Tris-HCl buffer, pH 7.0, 10 ml; 6-phosphogluconate (trisodium salt, Sigma) 250 mg; NADP, 20 mg; NBT, 20 mg; phenazine methosulfate, 2 mg; distilled water, 90 ml.

Differentiation of the species of the genus Bifidobacterium

The differential characteristics of the species of *Bifidobacterium* are indicated in Table 15.50. Other characteristics are cited in Tables 15.51–15.55.

List of species of the genus Bifidobacterium

1. **Bifidobacterium bifidum** (Tissier 1900) Orla-Jensen 1924, 472.[AL] (*Bacillus bifidus* Tissier 1900, 86.)

bi'fi.dum. L. neut. adj. *bifidum* cleft, divided.

Although the cells are highly variable in appearance, some traits observed in cells grown in TPY agar stabs are distinct (Fig. 15.96*A*). Groupings of "amphora-like" cells are characteristic. (Sundman et al., 1959).

Distinction of two serovars have been made; serovar *a* predominates in the feces of human adults while *b* predominates in that of neonates (variants *a* and *b*, Reuter, 1963; these variants differ in sucrose, melibiose and maltose fermentation).

Type strain: Ti (Tissier) (ATCC 29521).

2. **Bifidobacterium longum** Reuter 1963, 502.[AL]

long'um. L. neut. adj. *longum* long.

In TPY agar strains of this species show mostly very elongated and relatively thin cellular elements with slightly irregular contours and rare branchings (Fig. 15.96*B*).

In TPY broth most strains develop with uniform turbidity, clearing is slow and the sediment is viscous.

Two biovars are distinguished: biovar *a*, more frequent in human adults and slowly fermenting mannose and biovar *b*, more frequent in neonates and mannose negative (Reuter, 1963). *B. longum* subsp. *animalis*, with two biovars *a* and *b*, was distinguished from *B. longum* subsp. *longum* on the basis of melezitose and mannose fermentation and ecology (Mitsuoka, 1969, 60). The biovars *a* and *b* have been recognized on DNA-homology as belonging to the species *B. animalis* Scardovi and Trovatelli, and to *B. pseudolongum* Mitsuoka respectively (Scardovi et al., 1971b; Scardovi and Trovatelli, 1974).

B. longum is apparently the only species among those usually found in human feces which possesses a large variety of plasmids (Sgorbati et al., 1982).

On DNA-homology values (50–78%), this species is most closely related to *B. infantis* (see further comments under *B. infantis*).

Type strain: ATCC 15707 (E 194 b, from feces of a human adult, Reuter, 1971).

On DNA homology values (50–70%) this species is most closely related to *B. longum*.

Type strain: ATCC 15697 (S 12, from feces of human infant, Reuter, 1971).

Comments on the identification of *B. longum* and *B. infantis*. Arabinose is reported as not fermented by *B. infantis* ("*B. liberorum*" and "*B. lactentis*") while *B. longum* characteristically ferments melezitose (Reuter, 1963; Mitsuoka, 1969). Numerous strains isolated from infants' feces, which fermented arabinose or failed to ferment melezitose, were recognized by means of DNA-DNA hybridization as *B. infantis* and *B. longum*, respectively, (Scardovi, unpublished). The figures of DNA homology reported in Table 15.52 were obtained with these strains.

Isozyme patterns for transaldolase and 6PGD were determined in 63 and 126 strains allotted on DNA homology to *B. infantis* and *B. longum*, respectively: 90% of strains of *B. infantis* had the transaldolase (isozyme 5; migration 100) more anodal than that possessed by 72% of *B. longum* strains (isozyme 8, migration 90) (Scardovi et al., 1979a).

The electrophoretic patterns of soluble proteins distinguished the two species (Biavati et al., 1982).

Bifid strains isolated from the feces of the suckling calf could not be referred to either species, because they were more than 80% related on DNA homology both to *B. infantis* and *B. longum* reference strains; conversely, *B. infantis* and *B. longum* were not distinguishable when their DNA was annealed to that of the calf strains (Scardovi, unpublished). Although electrophoresis revealed that all these strains possess an "intermediate" form of transaldolase (isozyme 6, migration 96) (Scardovi et al., 1979a) and a distinct total proteins pattern (Biavati et al., 1982), no doubt the boundaries between the two species *B. longum* and *B. infantis* are tending to disappear. However 70% of the strains genetically *B. longum* carry a large array of plasmids whereas none of the strains of either *B. infantis* or "intermediates" from the calf do so, although strains of both species were isolated from the same specimens (Sgorbati et al., 1982). Pending studies on the possible taxonomic and evolutionary significance of plasmids in bifidobacteria, it is advisable to keep these two taxonomic entities separate, so diverse are they in their extrachromosomal DNA complements.

The following rule can be adopted: strains not fermenting arabinose should be retained as *B. infantis* while strains fermenting both arabinose and melezitose should be retained as *B. longum*; strains fermenting arabinose (and xylose) but not melezitose are *B. infantis* (reference strain B1269, ATCC 27920) if their transaldolase migrates more anodically (isozyme 5, migration 100) or *B. longum* (reference strain E194b, ATCC 15707) if their isozyme is less anodal (isozyme 8, migration 90); strains with an isozyme moving to an intermediate position (isozyme 6, migration 96) should be considered as "intermediates" (reference strains VT29 and VT42 from Collection of the Institute for Agricultural Microbiology, University of Bologna).

3. **Bifidobacterium infantis** Reuter 1963, 502.[AL]

in.fant' is. L. n. *infantis* an infant; M.L. gen. n. *infantis* of an infant.

Cellular morphology does not present specific traits so that it is similar to that of many other species of the genus (Fig. 15.98*D*).

These bifidobacteria do not ferment pentoses; found as predominant forms in breast-fed infant feces, distinguished biochemically and serologically and allotted to the species, *B. infantis* by Reuter (1963).

Bifid strains of the same ecology but fermenting xylose, were separated and distributed, on the basis of some differences in other sugars fermented, into two additional species, namely "*B. liberorum*" and "*B. lactentis*," (Reuter, 1963). DNA-homology studies indicated later that these two species were identical to *B. infantis* (Scardovi et al., 1971b).

"*Bifidobacterium parabifidum*" (Weiss and Rettger) Kandler and Weiss 1974, 40, is here considered as a synonym of *B. infantis*, because strain ATCC 17930 (strain Timberlain isolated by Norris et al., 1950, and studied by Pine and Howell, 1956, under the label 308), displayed (a) 82% DNA homology with ATCC 27920, one of the reference strains of *B. infantis*; (b) 76% DNA homology with ATCC 15707, type strain of *B. longum*; and (c) possessed the proteins pattern of *B. infantis* (Biavati et al., 1982).

4. **Bifidobacterium breve** Reuter 1963, 502.[AL]

bre've. L. neut. adj. *breve* short.

The cell morphology suggests the specific epithet; the thinnest and shortest cells among bifids found in the human intestine (Fig. 15.96*C*).

Among the bifids not fermenting pentoses isolated from newborn infants, the rank of species was attributed to those fermenting mannitol and sorbitol; two biovars *a* and *b* different towards melezitose were

recognized (Reuter, 1963). Serologically related strains isolated from breast-fed older infants and which did not ferment mannitol or sorbitol were referred to a separate species, namely "*B. parvulorum*" (Reuter, 1963).

Strains S50 (ATCC 15698), type strains of "*B. parvulorum*" biovar *a*, strain S17c (ATCC 15699) "*B. parvulorum*" biovar *b*, and strain S (ATCC 15701) *B. breve* biovar *b*, were 88, 94 and 86%, respectively, related on DNA homology to strain S1 (ATCC 15700), type strain of *B. breve* biovar *a*; other DNA homology tests proved the genetic identity of the two Reuter's species (Scardovi et al., 1971b).

On the basis of DNA homology *B. breve* is more closely related to both *B. infantis* and *B. longum* than to any other species of the genus (40-60% homology).

One hundred and six strains of *B. breve*, including a few strains fermenting arabinose and xylose, all identified by DNA-DNA homology test, were studied for their transaldolase and 6PGD isozymes: Fifty-seven percent of the strains had unique zymograms and 25% displayed some zymogram as *B. coryneforme*, a bifid found only in the intestine of the honey bee (Scardovi et al., 1979a).

Type strain: ATCC 15700 (S1 from feces of human infant, Reuter, 1971).

6. Bifidobacterium adolescentis Reuter 1963, 502.[AL]

a.do.les.cen'tis. L. n. *adolescens*: M.L. gen. n. *adolescentis* of an adolescent.

The cellular morphology is common to that of many other species of the genus (Fig. 15.98*D*). Reuter (1963) named as *B. adolescentis* those pentose-fermenting bifidobacteria he first found to predominate, along with *B. longum*, in the feces of human adults. Four biovars, *a*, *b*, *c* and *d* varied in fermentation of sorbitol and mannitol, and were serologically distinct (Reuter, 1963).

Among those species which are regularly found in man, *B. adolescentis* occurs most frequently in sewage (Scardovi et al. 1979a; Scardovi, unpublished). Reuter's biovars *b* and *d*, namely those which do not ferment sorbitol, cannot be distinguished phenotypically from *B. dentium* (see Table 15.51); however, transaldolase isozymes migrate differently, i.e. *B. dentium* isozyme 4 migrates 100, while that of *B. adolescentis* (number 8) migrates 87 (Scardovi et al., 1979a).

The PAGE procedure can be used alternatively (Biavati et al. 1982).

Type strain: ATCC 15703 (E194a from feces of human adult, Reuter, 1971).

6. Bifidobacterium angulatum Scardovi and Crociani 1974, 19.[AL]

an.gu.la'tum. L. Part. adj. *angulatus* with angles, angular.

Cells grown in TPY agar stabs generally and characteristically disposed in V (angular) or palisade arrangements similar to corynebacteria; rarely enlarged at the extremities; branching absent. This morphological type is unique among bifidobacteria (Fig. 15.96*D*).

Anaerobic but more sensitive to O_2 than most bifidobacteria (measured by the depth of growth in stabs). CO_2 does not affect this sensitivity but it strongly enhances anaerobic growth.

First isolated from adult human feces and then found in sewage.

Type strain: ATCC 27535 (B677, from feces of human adult, Scardovi and Crociani, 1974).

Twenty percent of strains of *B. angulatum* ferment sorbitol and can thus readily be distinguished from other species fermenting this sugar (Table 15.55). Most strains do not ferment sorbitol and so could be confused (in the case of doubtful morphology) with *B. globosum*, *B. pseudolongum* and sorbitol-negative strains of *B. pseudocatenulatum* from calf feces (nearly half of the strains from this source are inactive toward sorbitol; Scardovi et al. 1979 b). *B. angulatum* possesses the least anodal transaldolase (number 5) in respect of the named taxa.

7. Bifidobacterium catenulatum Scardovi and Crociani 1974, 18.[AL]

ca.te.nu.la'tum. M.L. adj. *catenulatum* having small chains.

Cells grown in TPY agar stabs are generally and characteristically arranged in chains of three, four or more globular elements. The distal ends of the chains are usually tapered. Distinct branchings, club-like swellings or spatula-like extremities are rare (Fig. 15.96*E*)

Anaerobic. CO_2 without effect on O_2 sensitivity or anaerobic growth.

Riboflavin and pantothenate required for growth.

Although most strains of *B. catenulatum* ferment sorbitol but not mannitol similar to biovar c of *B. adolescentis* and *B. pseudocatenulatum* (see below), they can be distinguished from the former because they ferment melezitose and from the latter because they do not ferment starch (see Table 15.55).

Found in feces of human adult and in sewage.

The mol% G + C of the DNA is 55 (T_m). This is the lowest value found in bifidobacteria.

Type strain: ATCC 27539 (B669 from feces of human adult, Scardovi and Crociani, 1974).

Most strains of the species show DNA relatedness to *B. adolescentis* (reference DNA from strain E298b ATCC 15705, biovar c of Reuter) in the range 30–57% (Scardovi and Crociani, 1974): *B. catenulatum* should be considered (as also should *B. pseudocatenulatum*, see below) to be more related to *B. adolescentis* than to any other species of the genus on this basis.

8. Bifidobacterium pseudocatenulatum Scardovi, Trovatelli, Biavati and Zani 1979, 309.[AL]

pseu.do.ca.te.nu.la'tum. Gr. adj. pseudes false; L. adj *catenulatum* specific epithet; M.L. adj. *pseudocatenulatum* the false (*B.*) *catenulatum*.

Cell morphology is one of the most variable among bifidobacteria in that it shows highly diverse traits according to strain and origin; (Scardovi et al. 1979b).

Anaerobic; CO_2 is without effect upon sensitivity or anaerobic growth.

Riboflavin, pantothenate and nicotinic acid required for growth (Table 15.53).

Sorbitol is fermented by all strains found in the feces of infants and by 50% of those found in calf feces. The sorbitol fermenting strains can be distinguished from *B. adolescentis* strains because they are melezitose-negative and from *B. catenulatum* because they invariably ferment starch. The sorbitol-negative strains of this species (so far found only in calf feces) can be distinguished from *B. globosum* or *B. pseudolongum* species also found in calf feces, and from *B. angulatum*, not only on the basis of different morphology, but also by means of their transaldolase zymogram (see Table 15.50).

The mol% G + C of DNA is 57.5 (T_m) (see Table 15.50).

The DNA of this species is 46–68% related to that of *B. catenulatum* but is virtually unrelated to that of any other species of the genus (see Table 15.52; Scardovi et al. 1979b).

The structure of the interpeptide bridge of cell wall peptidoglycan is shared by *B. catenulatum* and *B. angulatum* (see Table 15.50).

Found abundantly in sewage, in the feces of breast- and bottle-fed infants and in the feces of suckling calves.

Type Strain: ATCC 27919 (B1279 from feces of human infant, Scardovi et al., 1979b).

Differentiation of *B. catenulatum* from *B. pseudocatenulatum*. There is no doubt that these two species are closely related as indicated by their DNA homology. However, the G + C content of their DNA differs by 3 mol% (see Table 15.50), the same difference which exists between *B. globosum* and *B. pseudolongum* (see below); none of the strains shown genetically to be *B. catenulatum* ferments starch or mannose, whereas strains recognized by DNA homology as *B. pseudocatenulatum* ferment these compounds; *B. catenulatum* does not require nicotinic acid for growth as does *B. pseudocatenulatum*; furthermore, *B. catenulatum* has so far never been isolated from the feces of suckling calves.

Forty-one strains of *B. catenulatum* and 120 strains of *B. pseudocatenulatum* were studied for their transaldolase and 6PGD isozymes content. The isozymes of these two species migrate very differently (see Table 15.50); the few *B. pseudocatenulatum* strains (7 out of 120) which possess a transaldolase electrophoretically identical to that of *B. catenulatum* (number 5), display a much more anodal 6PGD isozyme

Characteristics differentiating the species of the genus Bi]br[illegible]erium

Species	Mol% G + C of DNA[a]	Murein type[b]	Electrophoretic patterns of enzymes[c]			Immunological specificity group[f]	Cell morphology	Found in[g]
			Transaldolase	6PGD[d]	F6PPK[e]			
1. *B. bifidum*	$58_{(Td)}$	Orn(Lys)-D-Ser-D-Asp	7	7-(8)	15	B	Distinct (Fig. 15.96*A*)	Feces of human infant and adult; human vagina; feces of suckling calf
2. *B. longum*	$58_{(Td)}$	Orn(Lys)-Ser-Ala-Thr-Ala	(5)-6-8	5-(6)	15	B		Feces of human adult and infant; (human clinical)
3. *B. infantis*	$58_{(Td)}$	Orn(Lys)-Ser-Ala-Thr-Ala	5-(6)-(8)	(3)-4-(5)	15	B		Feces of human infant; (human vagina)
4. *B. breve*	$58_{(Td)}$	Lys-Gly	6	(5)-6-6_a-7	15	B	Distinct (Fig. 15.96*C*)	Feces of human infant and adult; (human vagina and clinical)
5. *B. adolescentis*	$58_{(Td)}$	Lys(Orn)-D-Asp	8	5	15	B		Feces of human adult; sewage; (rumen of cattle; feces of monkey and dog)
6. *B. angulatum*	$59_{(Tm)}$	Lys(Orn)-D-Asp	5	5	15	A	Distinct (Fig. 15.96*D*)	Feces of human adult; sewage
7. *B. catenulatum*	$55_{(Tm)}$	Lys(Orn)-Ala_2-$Ser_{0.2-1.0}$	5	6–8	15	A	Distinct (Fig. 15.96*E*)	Feces of human adult and infant; (sewage)
8. *B. pseudocatenulatum*	$57.5_{(Tm)}$	Lys(Orn)-Ala_2-$Ser_{0.2-1.0}$	4-(5)	1–3		A		Feces of human infant and suckling calf; sewage
9. *B. dentium*	$61_{(Tm)}$	Lys(Orn)-D-Asp	4	(2)[d]	15	A		Human dental caries and clinical (feces of human adult and infant; human oral cavity and vagina)
10. *B. globosum*	$64_{(Tm)}$	Orn(Lys)-Ala_{2-3}	2	(3_a)-(4)-(5)-6-(7)	10	E		Feces of pig, suckling calf, rat, rabbit and lamb; rumen of cattle; (sewage)
11. *B. pseudolongum*	$60_{(Tm)}$	Orn(Lys)-Ala_{2-3}	2	7	10	E		Feces of chicken, cattle, rat and mice
12. *B. cuniculi*	$64_{(Tm)}$	Orn(Lys)-Ser(Ala)-Ala_2	1	4		C	Distinct (Fig. 15.97*C*)	Feces of rabbit
13. *B. chocrinum*	$66_{(Tm)}$	Orn(Lys)-Ser(Ala)-Ala_2	3	4				
14. *B. animalis*	$60_{(Tm)}$	Orn(Lys)-Ser(Ala)-Ala_2	6	8-9	10		Distinct (Fig. 15.97*B*)	Feces of piglet; (sewage)
15. *B. thermophilum*	$60_{(Tm)}$	Orn(Lys)-D-Glu	(7)-8	7-8-9-(9_a)	10	D		Feces of rat, chicken, rabbit, calf; sewage
16. *B. boum*	$60_{(Tm)}$	Lys-D-Ser-D-Glu	6	8-9-9_a		D		Feces of pig, piglet, chicken, calf, rumen of cattle; sewage
17. *B. magnum*	$60_{(Tm)}$	Lys(Orn)-Ala_2-$Ser_{0.2-1.0}$	5	7	10	C′	Distinct (Fig. 15.97*D*)	Rumen of cattle; feces of piglet
18. *B. pullorum*	$67_{(Tm)}$	Lys(Orn)-D-Asp	2	Absent[d]	10		Distinct (Fig. 15.97*A*)	Feces of rabbit
19. *B. suis*	$62_{(Tm)}$	Orn(Lys)-Ser-Ala-Thr-Ala	6	5-8	10	G		Feces of chicken
20. *B. minimum*	$61.5_{(Tm)}$	Lys-Ser	10	6	10	F	Distinct (Fig. 15.97*F*)	Feces of piglet
21. *B. subtile*	$61.5_{(Tm)}$	Lys(Orn)-D-Asp	3	2	10–15	F	Distinct (Fig. 15.97*E*)	Sewage

22. *B. coryneforme*		Lys(Orn)-D-Asp	6	6	16		Distinct (Fig. 15.98C)	Intestine of *Apis mellifera* L. subsp. *mellifera*
23. *B. asteroides*	$59_{(Tm)}$	Lys-Gly	(6):(7)-(7_a)-8-(8_a)-(8_b)-(9)-(9_a)	(9)-(9_a)-(9_b)-(10)-10_a-10_b-(11)-(12)-(13)	16	H	Distinct (Fig. 15.98A)	Intestine of *A. mellifera* L. subspp. *mellifera*, *ligustica* and *caucasica*; (*A. cerana* F.)
24. *B. indicum*	$60_{(Tm)}$	Lys(Orn)-D-Asp	(6)-7-8-9	6-6_a-(7)-8-(9)-(9_a)-(9_b)	16	H	Distinct (Fig. 15.98B)	Intestine of *A. cerana* F. and *A. dorsata* F.

[a] Data based on buoyant density (Bd) were taken from Gasser and Mandel, 1968; for the other values (T_m) see under single species description.
[b] Taken from Kandler and Lauer, 1974, or kindly provided by O. Kandler, Institute of Botany, University of München, Germany.
[c] Taken from Scardovi et al., 1979a; 6PGD = 6-phosphogluconic dehydrogenase ($NADP^+$). Numbers 1 to 10 and 1 to 13 were given to isozymes of transaldolase and 6PGD, respectively, in the order of decreasing anodic mobility (suffixed numbers indicate additional isozymes). Boldface numbers are the isozymes of the type strains. Numbers in parentheses are isozymes found in less than 10% of the strains studied. 6PGD in undetectable by spot-staining in most strains of *B. dentium* and in all strains of *B. pullorum*.
[d] Taken from Scardovi et al., 1971a; F6PPK = fructose-6-phosphate phosphoketolase. Numbers indicate the migration relative to that of *B. globosum* taken = 10. Phosphoketolases of migration 15 and those of migration 10 were ecologically distinguished as "human" and "animal" type, respectively (Sgorbati et al., 1976).
[e] Taken from Sgorbati and London, 1982.
[f] Sources are listed in the order of decreasing frequency of occurrence of the species therein. Sources in parentheses are occasional.

(Table 15.50), Scardovi et al., 1979a). Although some data suggest the existence of "intermediate" strains (Scardovi et al., 1979b) like those found between *B. longum* and *B. infantis* (see above) and, although PAGE procedure gave uncertain results (Biavati et al., 1982), it is advisable at present to maintain the rank of species for these two taxa.

9. Bifidobacterium dentium Scardovi and Crociani 1974, 18.^{AL}

den'tium. L. mas. n. *dens* tooth; L. plural gen. n. *dentium* of teeth.

Cells grown in TPY agar show a general morphology resembling that of *B. infantis* (Fig. 15.98*D*) and, hence, are without any characteristic appearance.

Anaerobic: CO_2 does not affect sensitivity to O_2 or anaerobic growth.

A number of bifid strains isolated from human dental caries, feces of human adult and human vagina, phenotypically assigned to *B. adolescentis* were first recognized as forming a distinct "dentium" DNA homology group, together with strains isolated from the oral cavity (Beerens et al., 1957) and strain 3859 labeled *B. appendicitis*. The species *B. dentium* (Table 15.52) has some DNA relatedness to *B. adolescentis*, but is related less or not at all to other species of the genus. DNA of strains *Actinomyces eriksonii* ATCC 15423 and ATCC 15424 are completely homologous to that of *B. dentium* reference strain B764 (Scardovi et al. 1979a).

B. dentium requires riboflavin and pantothenate for growth, similar to *B. angulatum* and *B. catenulatum*.

Type strain: ATCC 27534 (B764, from human dental caries, Scardovi and Crociani, 1974).

Comments on the identification of *B. dentium*. *B. dentium* cannot be distinguished phenotypically from biovars *b* and *d* of *B. adolescentis* (strains not fermenting sorbitol, see Table 15.55); hence the distinction between these two species should be based either on transaldolase isozymes or on electrophoresis patterns of total cellular proteins (see under *B. adolescentis*).

10. Bifidobacterium globosum (ex Scardovi, Trovatelli, Crociani and Sgorbati 1969) Biavati, Scardovi and Moore 1982, 368.^{VP} Considered (Rogosa, 1969) as a synonym of *B. pseudolongum* Mitsuoka; not reported in the Approved Lists (1980); its revival proposed by Biavati et al. (1982).

glo.bo'sum. from L. n. *globus* ball, sphere.

Cells grown anaerobically in TPY agar are generally short, coccoid or almost spherical to curved or tapered, arranged singly or doubly or rarely in short chains (Fig. 15.96*F*). This morphology, shared by *B. pseudolongum* Mitsuoka, does not change with strain or source; only cells grown in air + CO_2 have bifurcations or short cross-branchings, often with enlarged ends.

Anaerobic aerotolerant organisms. CO_2 is without effect on anaerobic growth but permits development in high O_2 tensions (slope incubated in 90% air + 10% CO_2). Aerobically grown cells do not show catalase or catalase-like activity (hemin).

Pantothenate, riboflavin, thiamin and folic acid required for anaerobic growth. Ammonia satisfies nitrogen requirements.

Initially the strains allotted to this species were isolated from the bovine rumen; they did not ferment xylose, mannose and cellobiose, and only rarely arabinose (Scardovi et al., 1969). Pentose-fermenting strains were subsequently isolated from piglets and recognized as *B. globosum* on DNA homology (Zani et al., 1974). Many other strains genetically assigned to *B. globosum* were later isolated from feces of various animals and included strains fermenting mannose, cellobiose or mannitol as well as some slowly fermenting or inactive toward fructose (Scardovi et al., 1979a).

Intraspecific DNA homologies ranged from 78–106%: relatedness to reference of *B. pseudolongum* varied in the range 69–73% (Scardovi et al., 1971b). Range 50–67% of homology was observed between *B. globosum* and *B. cuniculi* (Table 15.52).

Unlike most species of the genus, the enzyme fructose-bisphosphate aldolase and both the hexose monophosphate (HMP) dehydrogenases can be detected in cell-free extracts. Functioning of aldolase in intact cells was proved by the expected increase of acetate by degrading

Table 15.51.
Fermentative characteristics distinguishing the species of the genus Bifidobacterium[a]

	D-Ribose	L-Arabinose	Lactose	Cellobiose	Melezitose	Raffinose	Sorbitol	Starch	Gluconate
1. *B. bifidum*	−	−	+	−	−	−	−	−	−
2. *B. longum*	+	+	+	−	+	+	−	−	−
3. *B. infantis*	+	−	+	−	−	+	−	−	−
4. *B. breve*	+	−	+	d	d	+	−	−	−
5. *B. adolescentis*	+	+	+	+	+	+	d	−	−
6. *B. angulatum*	+	+	+	−	−	+	d	+	+
7. *B. catenulatum*	+	+	+	+	−	+	d	+	d
8. *B. pseudocatenulatum*	+	+	+	d	−	+	+	−	d
9. *B. dentium*	+	+	+	+	+	+	d	+	d
10. *B. globosum*	+	d	+	−	−	+	−	+	+
11. *B. pseudolongum*	+	+	d	d	d	+	−	+	−
12. *B. cuniculi*	−	+	−	−	−	−	−	+	−
13. *B. choerinum*	−	−	+	−	−	+	−	+	−
14. *B. animalis*	+	+	+	d	d	+	−	+	−
15. *B. thermophilum*	−	−	d	d	d	+	−	+	−
16. *B. boum*	−	−	d	−	−	+	−	+	−
17. *B. magnum*	+	+	+	−	−	+	−	+	−
18. *B. pullorum*	+	+	−	−	−	+	−	−	−
19. *B. suis*	−	+	+	−	−	+	−	−	−
20. *B. minimum*	−	−	−	−	−	+	−	−	−
21. *B. subtile*	+	−	−	−	+	+	+	+	+
22. *B. coryneforme*	+	+	−	+	−	+	−	−	+
23. *B. asteroides*	+	−	+	−	−	+	−	−	d
24. *B. indicum*	+	−	+	−	+	−	−	−	+

[a] Symbols: see Table 15.25.

glucose in the presence of iodoacetic acid (Schramm et al., 1958; Scardovi and Trovatelli, 1969). Glucose-6-phosphate dehydrogenase, undetectable in some strains, was found to be either NAD^+ or $NADP^+$ dependent (Scardovi and Sgorbati, 1974).

Zymograms of 103 strains of *B. globosum* were examined. All possessed the transaldolase isozyme 2 and more than 80% contained the 6PGD isozyme 6; four other 6PGD isozymes were found (Scardovi et al., 1979a: Table 15.50).

PAGE proteins patterns of selected strains from various sources were identical and quite distinct from those of any other species of the genus, including those of the most closely related species, *B. pseudolongum*, thus further indicating the validity of the species *B. globosum*, the revival of which was proposed by Biavati et al. (1982).

Plasmids of large molecular weights (13.5, 24.5 and 46 MDa) were found in 22% of *B. globosum* strains studied (Sgorbati et al., 1982). The mol% G + C of the DNA is 63.8 ± 0.4 (T_m) (Scardovi et al., 1971b). Originally isolated from the bovine rumen (Scardovi et al., 1969), later found in the feces of the piglet, rat, lamb, chicken, rabbit, calf, sewage and in a single specimen of feces of the human infant (Scardovi et al., 1979a).

Type strain: ATCC 25865 (RU 224 from bovine rumen, Scardovi et al., 1969).

11. Bifidobacterium pseudolongum Mitsuoka 1969, 60.[AL]

pseu.do.long'um. Gr. adj. *pseudes* false; L. adj. *longum* specific epithet; M.L. neut. adj. *pseudolongum* false (*B.*) *longum*.

Cells grown in TPY agar are morphologically identical to those of *B. bosum*.

The relations to O_2 and CO_2 have not been studied in detail. Requirements for growth factors are unknown.

Mitsuoka (1969) recognized as belonging to this species bifid strains ated from a variety of animals; these strains fermented arabinose, ose, starch and glycogen but slowly fermented fructose, thus differ- from strains of *B. thermophilum* isolated from the same sources tsuoka, 1969). Four biovars, *a*, *b*, *c* and *d* were recognized on the s of differences in the fermentation of mannose, lactose, cellobiose melezitose (Mitsuoka, 1969).

Fermentative characters correspond to those reported for *B. globosum* (see Tables 15.51 and 15.54).

The presence of fructose-bisphosphate aldolase or glucose-6-phosphate-dehydrogenase in cell-free extracts was not investigated.

On electrophoresis, the type strain PNC-2-9G (ATCC 25526 biovar *a*), strain 29-Sr-T representative of biovar *c* and strain Mo-2-10 representative of biovar *d*, all displayed the transaldolase isozyme 2 and the 6PGD isozyme 7, i.e. a pattern identical to that possessed by only two *B. globosum* strains of the 103 studied (Table 15.50; Scardovi et al., 1979a).

PAGE proteins patterns of some strains, including type strain ATCC 25526, were clearly distinct from those of *B. globosum* (Biavati et al., 1982).

With DNA homology, *B. pseudolongum* is related 65–70% to *B. globosum* and 10–15% to *B. cuniculi*. The Mitsuoka strain C10-45 (*B. longum* subsp. *animalis* biotype *b*, Mitsuoka 1969) is completely homologous to strain Mo-2-10 representative of biovar *d* of *B. pseudolongum* (Scardovi et al., 1971b).

Plasmids were absent in three strains studied (Sgorbati et al., 1982).

The mol% G + C of the DNA is 60.3 ± 0.45 (Scardovi et al., 1971b).

Type strain: ATCC 25526 (PNC-2-9G from feces of swine, Mitsuoka, 1969).

Comments on the differentiation between *B. globosum* and *B. pseudolongum*. Phenotypic characters cannot be used to distinguish between these two species. The interpeptide bridge of the cell wall peptidoglycan has the same structure. A similar ecological distribution is shared by the two species. Their taxonomic separation is based on (a) different content of G + C mol% of their DNA, (b) their reciprocal DNA homology and the DNA homology to other related species, (c) distinct PAGE total cellular proteins patterns, (d) differences in 6PGD zymograms and (e) differences in plasmid cellular complement. On DNA homology, *B. globosum* is much more closely related to *B. cuniculi* (50–67%) than is *B. pseudolongum* (8–16%) (Table 15.52). Differences in isozyme and plasmid content, even if biased by the small number of strains of *B. pseudolongum* investigated, are striking.

As indicated in Table 15.55, the practical distinction between the two species can be based on electrophoresis of the 6PGD dehydrogen-

Table 15.52

DNA homology relationships[a] of the species of genus **Bifidobacterium**

Competitor DNA from	Percent homology[b] to reference DNA from:[c]																							
	1. *B. bifidum*	2. *B. longum*	3. *B. infantis*	4. *B. breve*	5. *B. adolescentis*	6. *B. angulatum*	7. *B. catenulatum*	8. *B. pseudocatenulatum*	9. *B. dentium*	10. *B. globosum*	11. *B. pseudolongum*	12. *B. cuniculi*	13. *B. choerinum*	14. *B. animalis*	15. *B. thermophilum*	16. *B. boum*	17. *B. magnum*	18. *B. pullorum*	19. *B. suis*	20. *B. minimum*	21. *B. subtile*[c]	23. *B. asteroides*[c]	24. *B. indicum*
1. *B. bifidum*	100	2	20–28	10–20	0		25	15		0		10	27	10	5[d]	11	5	20	0	4	4	0	0
2. *B. longum*	*40*	75–101	50–76[e]	12	0		*20*	22		0		17	16	6	0[d]	23	0–29	9	0[f]	7	23	0	
3. *B. infantis*	42	50–79	74–101	17–50			*20*	15		0		16–23	20–22	15[g]	5[d]	11	15[g]	13	0	4[g]	5[g]	0	
4. *B. breve*	25	40	40	100			*24*	10		0		12	21	20	5[d]	18	10	18	0	10	17	0	
5. *B. adolescentis*	*14*		22	5–10	70–102	20–44	20–57	30	24–49			25	20	30		25	14	21		5	13		
6. *B. angulatum*	*24*	6–13	20[h]	12	20–30	76–100	20–35	20	8–20	20		17	16	11	18[d]	10	5	0	20	23	8		
7. *B. catenulatum*	20	0–26	15[h]	10	22–57	2–37	78–101	50–80[e]	3–48	5		10	32	10	20[d]	8	17	12	25	30	12		
8. *B. pseudocatenulatum*							46–88	78–115				10	32			5							
9. *B. dentium*	20	0–13	20[h]	12	15–57	5–26	16–45	18	69–110	12		17	15	6	20[d]	0	8	0	20	5	0		
10. *B. globosum*	29		*25*	25				5		100	75[e]	45	50	40	14[d]	14	32	16	10	35	10	13	12
11. *B. pseudolongum*	*35*		*17*	*7*				5		65–70	100	10–15	45–50			10			9–27				
12. *B. cuniculi*	*7*		*17*	22				10		50–67	8–16	94–102	9–15	18–25	12–20[d]	5		2					
13. *B. choerinum*	*32*		*8*	*15*				25		26–57	37–62	8–20	75–120	4–20	15–35[d]	20		4					
14. *B. animalis*	10	8	10[h]	20	25	10	10	10	4	30	34	31–26	19	72–100	25[d]	21	15	0	20	5	10	5	0
15. *B. thermophilum*	22		29	10–22				16[i]		6[i]	0–23	15–25	15–20	23[i]	79–117	27–80[e]	11[i]	10	6–12	4[i]	15[i]	0[i]	0[i]
16. *B. boum*	*38*		7	23				5				8	23	*11*	36–74	69–96		0					
17. *B. magnum*	10	4	25	9	6	5	26	20	21	6		10	10	23	20[d]	12	75–106	2	9	20	7		
18. *B. pullorum*	27		*39*	33				12				25	37	*35*		13		94–107					
19. *B. suis*	28		29	22				25		23	0	21	21	*5*	10[d]	7	10	10	100	10	8		
20. *B. minimum*	5	10	6[h]	10	4	20	25	25	0	5		23	36	5	10[d]	0	19	8	7	100–103	16–20	3	0
21. *B. subtile*	5	20	5[h]	14	15	11	8	10	0	8		14	34	6	18[d]	0	0	9	11	10–31	70–100	5	4
22. *B. coryneforme*				*6*						5		20	36	0				0		4	0	23	60
23. *B. asteroides*				*0*				5		0		11	60		0[d]	0		6	0	0	2	100	30
24. *B. indicum*				11						0		20	44		10[d]			17		0	0	23	100

[a] DNA immobilized to filter and the single-point competition procedure of Johnson and Ordal (1968) were used throughout.

[b] Data from Scardovi et al., 1970; Scardovi et al., 1971b; Scardovi and Trovatelli, 1974; Scardovi and Zani, 1974; Scardovi and Crociani, 1974; Scardovi et al., 1979b; Trovatelli et al., 1974; Scardovi, unpublished (italicized values).

[c] Species 22. *B. coryneforme* was not used as reference: the DNA from this species (type strain) was used as competitor. Type strains were generally used as source of reference DNA except for *B. longum*, *B. infantis*, *B. catenulatum*, *B. pseudocatenulatum*, *B. thermophilum* and *B. boum*: in these cases more than one strain were used as source of reference DNA.

[d] DNA from strain RU326 (ATCC 25866) (*B. ruminale*) used as reference.

[e] Groups of most closely related species (*boxed*). Large numbers and more than one strain used as competitor and reference respectively. Results are reported as ranges of the obtained values.

[f] DNA from *B. longum* strain B816 isolated from human vagina used as competitor (Scardovi et al., 1970).

[g] DNA from strain S76e Reuter (ATCC 15702) (*B. liberorum*) used as competitor.

[h] DNA from strain S76e Reuter (ATCC 15702) (*B. liberorum*) used as reference.

[i] DNA from strain RU326 (ATCC 25866) (*B. ruminale*) used as competitor.

Table 15.53.
Vitamin and growth factor requirement of some species of the genus **Bifidobacterium**[a,b]

Species	Riboflavin	Pantothenate	Nicotinic acid	Pyridoxine	Thiamine	Folic acid	*p*-Aminobenzoic acid	Biotin	Tween 80
1. *B. bifidum*[c]	+	−	+	−	−	+	+	−	+[d]
3. *B. infantis*[e]	−	+	−	−	−	−	−	−	+
5. *B. adolescentis*[c]	+	+	+	+	+	−	−	−	−
6. *B. angulatum*[c]	+	+	−	−	−	−	−	−	−
7. *B. catenulatum*[c]	+	+	−	−	−	−	−	−	−
8. *B. pseudocatenulatum*[c]	+	+	+	−	−	−	−	−	−
9. *B. dentium*[c]	+	+	−	−	−	−	−	−	−
10. *B. globosum*[c]	+	+	−	−	+	+	−	−	−
15. *B. thermophilum*[c]	+	+	−	+	−	−	−	−	−
18. *B. pullorum*[c]	−	−	+	+	+	+	+	−	+
19. *B. suis*[c]	+	−	−	−	−	−	−	−	−
23. *B. asteroides*[f]	+	+	+	+	+	−	−	+	−

[a] Data from Trovatelli and Biavati (1980).
[b] Symbols: +, required; −, not required.
[c] Ten strains, type strains included, were studied from each species.
[d] Only stimulatory.
[e] Data from Matteuzzi et al. 1971.
[f] Data from Scardovi and Trovatelli, 1969.

ble 15.54.
Additional fermentation reactions in the genus **Bifidobacterium**[a]

Species	Xylose	Mannose	Fructose	Galactose	Sucrose	Maltose	Trehalose	Melibiose	Mannitol	Inulin	Salicin
1. *B. bifidum*	−	−	+[b]	+	d[c]	−[d]	−	d	−	−	−
2. *B. longum*	d	d	+	+	+	+	−	+	−	−	−
3. *B. infantis*	d	d	+	+	+	+	−	+	−	d	−
4. *B. breve*	−	+	+	+	+	+	d	+	d	d	+
5. *B. adolescentis*	+	d	+	+	+	+	d	+	d	d	+
6. *B. angulatum*	+	−	+	+	+	+	−	+	−	+	+
7. *B. catenulatum*	+	−	+	+	+	+	d	+	d	d	+
8. *B. pseudocatenulatum*	+	+	+	+	+	+	d	+	−	−	+
9. *B. dentium*	+	+	+	+	+	+	+	+	+	−	+
10. *B. globosum*	d	−	+[e]	+	+	+	−	+	−	−[f]	−[g]
11. *B. pseudolongum*	+	+[h]	+	+	+	+	−	+	−	−	−
12. *B. cuniculi*	+	−	−	+	+	+	−	+	−	−	−
13. *B. choerinum*	−	−	−	+	+	+	−	+	−	−	−
14. *B. animalis*	+	d	+	+	+	+	d	+	−	−	+
15. *B. thermophilum*	−	−[i]	+	+	+	+	d	+	−	d	d
16. *B. boum*	−	−	+	+[j]	+	+	−	+	−	+	−
17. *B. magnum*	+	−	+	+	+	+	−	+	−	−	−
18. *B. pullorum*	+	+	+	+[h]	+	+	+	+	−	+	+
19. *B. suis*	+	d[k]	d[k]	+	+	+	−	+	−	−	−
1. *B. minimum*	−	−	+	−	+	+	−	−	−	−	−
21. *B. subtile*	−	−	+	+	+	+	d	+	−	d	d
22. *B. coryneforme*	+	−	+		+	+	−	+	−		+
23. *B. asteroides*[l]	+[m]	−[n]	+	d	+	d	−	+	−	−	+
24. *B. indicum*[l]	−	d	+	d	+	d	−	+	−	−	+

[a] Symbols: see Table 15.25.
[b] Few strains do not ferment this sugar.
[c] When positive it is fermented slowly.
[d] Some strains ferment this sugar.
[e] Some strains are negative, especially from rat and rabbit feces.
[f] Some strains from piglets are positive.
[g] Some strains can ferment weakly.
[h] Generally delayed or slight fermentation.
[i] Some strains from sewage ferment this sugar.
[j] Some strains are weak fermenters.
[k] Reported "sometimes not fermented" (Matteuzzi et al., 1971).
[l] Sugars indicated "d" gave mostly erratic results.
[m] Few strains do not ferment pentoses.
[n] Few strains ferment this sugar.

ase: the isozymes of most strains of *B. globosum* are more anodal than those of *B. pseudolongum*. Alternatively, the total proteins electrophoresis should be used. With this method strains of *B. pseudolongum* fermenting melezitose (such as Mitsuoka's biovar *c*) can be promptly recognized as such instead of ascertaining the isozyme of transaldolase for their distinction from the phenotypically very similar *B. animalis* (see Table 15.55).

2. Bifidobacterium cuniculi Scardovi, Trovatelli, Biavati and 1979, 307.[AL]

cu.ni'cu.li. L. n. *cuniculus* rabbit; L. gen. n. *cuniculi* of the rabbit.

Cells grown in TPY agar stabs present a morphology very similar to that of *B. globosum* and *B. pseudolongum*. However, the short knobs or protuberances often in the center of the cells are rarely seen in other species (Fig. 15.97C).

Highly anaerobic. CO_2 has no effect on O_2 sensitivity or anaerobic growth.

Lactose and raffinose are characteristically not fermented. Fructose is regularly fermented but only in prereduced media.

Transaldolase isozyme 1 and 6PGD isozyme 4 were found in seven

Table 15.55.
Tabular key for the identification of the species of the genus **Bifidobacterium**[a]

Sorbitol	Arabinose	Raffinose	Ribose	Starch	Lactose	Cellobiose	Melezitose	Gluconate	6PGD isozymes[b]	Transaldolase isozymes[b]	Suggested species
+	+			+		+	+	+			*B. adolescentis*
							−				*B. pseudocatenulatum*
				−		−					*B. angulatum*
				−							*B. catenulatum*
	−				+						*B. breve*
					−						*B. subtile*
−										4	*B. dentium*
	+	+	+	+	+	+	+	+		8	*B. adolescentis*
								−		5	*B. animalis*
						−	−			4	*B. pseudocatenulatum*
						−	−		7	2	*B. pseudolongum*
									(3$_a$)-(4)-(5)-6-(7)	2	*B. globosum*
										5	*B. angulatum*
				−	+	−	+				*B. longum*
							−		(3)-4-(5)	5	*B. infantis*
									5-(6)	8	*B. longum*[c]
									7		*B. magnum*
									6		*B. coryneforme*
				−	+				9–13		*B. asteroides*
							−				*B. pullorum*
								−			*B. suis*
				−							*B. cuniculi*
		−									*B. breve*
	−	+	+	+	+						*B. infantis*
				−	+						*B. indicum*
					−					3	*B. choerinum*
				−						6	*B. boum*
										8	*B. thermophilum*
											B. bifidum
		−			+						*B. minimum*
					−						

[a] Symbols: see Table 15.25.
[b] Boldface numbers are the isozymes possessed by the type strains. Numbers in parentheses are the isozymes possessed by less than 10% of strains.
[c] Unlike *B. suis*, some strains of *B. longum* do not ferment ribose and display identical electrophoretic patterns.

strains studied by Scardovi et al., 1979. This pattern 1-4 is unique among bifidobacteria.

The DNA of the species is 50–67% related to that of *B. globosum* (reference strain RU230-ATCC 25864), but far less related to *B. pseudolongum* and other species of the genus.

The mol% G + C content of DNA is 64.1 ± 0.35 (T_m).

Found in feces of adult rabbit.

Type strain: ATCC 27916 (RA93 from feces of rabbit, Scardovi et al., 1979b).

Comments. B. cuniculi can be easily distinguished from the morphologically similar species *B. globosum*, *B. pseudolongum* and *B. animalis* which are also found frequently in rabbit feces, because unlike these species *B. cuniculi* does not ferment lactose, ribose or raffinose. The same fermentation pattern can be used to distinguish this species from the morphologically different *B. magnum*, another species isolated from the same source.

13. **Bifidobacterium choerinum** Scardovi, Trovatelli, Biavati and Zani 1979, 307.[AL]

choe.ri'num. M.L. adj. *choerinus* pertaining to a pig.

Cells grown in TPY agar stabs are often short or coccoid with morphology similar to that of *B. globosum*. In liquid medium cells may be elongated to 10–12 μm, bent and with rounded or spatulated ends.

Anaerobic. Effect of CO_2 not detectable.

The strains of this species which ferment raffinose but not ribose, arabinose and sorbitol cannot be distinguished on that basis from *B. thermophilum* and *B. boum* (see Table 15.55).

Transaldolase and 6PGD isozymes are 3 and 4 respectively; this pattern is unique among bifidobacteria.

The species has distinct PAGE proteins pattern (Biavati et al., 1982).

The mol% G + C of the DNA is 66.3 ± 0.15 (T_m).

The DNA of this species is related 26–57% and 37–62% to the DNA of *B. globosum* and *B. pseudolongum*, respectively. The DNA relatedness of 50% between *B. choerinum* and *B. asteroides* is unexpected.

Found in feces of piglet or, occasionally, in sewage.

Type strain: ATCC 27686 (Su806 from feces of pig, Scardovi et al., 1979b).

Comments. B. choerinum cannot be distinguished by sugar fermentation pattern from either *B. thermophilum* biovar c (Mitsuoka, 1969) or from strains of *B. boum* which ferment lactose but not melezitose (Table 15.51). These three species are frequently found in feces of piglets. The distinction between these species can be based on transaldolase electrophoresis: the most anodal isozyme is that of *B. choerinum* (migration 100), the least anodal that of *B. thermophilum* (isozyme 8· migration 84) and an intermediate form is that of *B. boum* (isozyme migration 90). Alternatively, PAGE patterns of soluble proteins can used.

14. **Bifidobacterium animalis** (Mitsuoka 1969) Scardovi and vatelli 1974, 26.[AL] (*Bifidobacterium longum* subsp. *animalis* (biot Mitsuoka 1969, 60.)

an.i.mal'is. L. gen. n. of an animal.

Bifids isolated from feces of the calf, sheep, rat and guinea pig, phenotypically very similar to *B. longum*, but inactive toward melezitose, were referred to a subspecies of *B. longum* (*Bifidobacterium longum* subsp. *animalis* Mitsuoka 1969, 60). Two biovars *a* and *b* were distinct: biovar *a* mannose-negative and biovar *b* mannose-positive (Mitsuoka, 1969). Mitsuoka's strains R101-8 biovar *a* (ATCC 25527) and C10-45 biovar *b* were not related to *B. longum* on DNA homology but one of them (C10-45 biovar *b*) was genetically *B. pseudolongum* (Scardovi et al., 1971b). Strain R101-8 was subsequently allotted to a DNA homology group of bifids isolated from chicken, rat, rabbit and sewage and proposed as a distinct species (Scardovi and Trovatelli, 1974).

Cells grown on TPY show characteristically the central portion slightly enlarged (Fig. 15.97*B*), branchings can occur to form cross-like aggregates of four cells distally inflated.

Anerobic. CO_2 has no effect upon O_2 sensitivity or anaerobic growth.

Transaldolase and 6PGD isozymes possessed by this species (isozyme 5 and 8 or 9, respectively) give a pattern shared with only few strains of *B. catenulatum* (see Table 15.50).

PAGE proteins pattern is distinct from that of all other species (P' 'ati et al., 1982).

- actose-bisphosphate aldolase and glucose-6-phosphate dehydrogenase demonstrable (Scardovi and Trovatelli, 1974).

'he mol% G + C of DNA is 60.1 ± 0.3 (T_m).

DNA unrelated to that of any other species of the genus.

Found in feces of rat, chicken, rabbit, calf and in sewage.

Type strain: ATCC 25527 (R101-8 from feces of rat, Mitsuoka, 1969).

Comments. B. animalis is readily differentiated from other species found in animal habitats and which ferment arabinose and xylose (namely, *B. globosum*, *B. pseudolongum*, *B. cuniculi*, *B. magnum*, *B. pullorum* and *B. suis*) by lactose and salicin fermentation: if a strain ferments both sugars it can be retained as *B. animalis* (*B. pullorum* among the listed species, ferments salicin but not lactose, see Table 15.54). "Human" species which ferment pentoses but not starch, such as *B. dentium* and *B. adolescentis*, can be distinguished from *B. animalis* by the absence of gluconate fermentation in *B. animalis* (see Table 15.55).

15. Bifidobacterium thermophilum Mitsuoka 1969, 59.[AL]

ther.mo'phil.um: Gr. n. *therme* heat; Gr. adj. *philus* loving; M.L. adj. *thermophilum* heat-loving.

Mitsuoka (1969) gave the specific epithet to strains of bifids not fermenting pentoses which he isolated from the feces of swine and ch'-'en, owing to their ability to grow at 46.5°C and to resist heating fo. 'min at 60°C. Scardovi et al. (1969) named strains not fermenting pentoses or lactose which he isolated from bovine rumen, as "*B.* - *inale.*" All experimental comparisons have demonstrated that these two species are identical (Scardovi et al., 1971b; Scardovi et al. 1979a; Biavati et al., 1982).

Cells grown in TPY agar stabs are long, slender, curved, arranged singly or in pairs, never in clumps; this morphology is shared by many other species of the genus.

Four biovars, *a*, *b*, *c* and *d* were distinguished by Mitsuoka (1969) according to differences in the fermentation of melezitose and lactose. Similar differences were found among strains isolated from the bovine rumen, feces of calf, sewage (Scardovi et al., 1979b) and feces of the piglet (Zani et al., 1974). A few strains genetically assigned to *B. thermophilum* which fermented arabinose and xylose, were found in sewage and in piglets (Scardovi et al., 1979b).

Both *B. globosum* and *B. thermophilum* can grow in 90% air + 10% CO_2 without the cells becoming catalase- or pseudo-catalase (hemin)-positive.

Growth factors required are riboflavin, pantothenate and pyridoxine; bases are not required; ammonia is an optimal source of nitrogen.

Fructose-bisphosphate aldolase and HMP dehydrogenases are always present in cell-free extracts (Scardovi et al., 1969). Aldolase was spot-tained after electrophoresis (Scardovi and Sgorbati, 1974).

DNA homology indicated 27–80% relatedness to *B. longum* but no relation to other species of the genus.

The mol% G + C of the DNA is 60 (T_m).

Type strain: ATCC 25525 (P2-91 from the feces of swine, Mitsuoka, 1969).

Comments. To distinguish between *B. thermophilum*, *B. boum* and *B. choerinum*, see under *B. choerinum*.

16. Bifidobacterium boum Scardovi, Trovatelli, Biavati and Zani 1979, 308.[AL]

bo'um. L. n. *bos* a cow; L. pl. gen. n. *boum* of cattle.

A few strains were isolated from the bovine rumen as morphovars of *B. thermophilum* (*B. ruminale*) (Scardovi et al., 1969); later their DNA was found to be nearly 70% related to *B. thermophilum* (Scardovi et al., 1971b). Subsequently, other strains from the bovine rumen were assigned to a DNA homology group IV, 55–75% related to *B. thermophilum* (Trovatelli and Matteuzzi, 1976).

From a large number of animal strains surveyed by DNA-DNA hybridization, 36 strains from the rumen and 5 from piglet feces were allotted to the new species *B. boum* (Scardovi et al., 1979b). Cells grown on TPY agar are in general more irregular than those of *B. thermophilum*, and vary greatly with strain. Most branched forms are seen in cells grown in air + CO_2.

Although *B. boum* does not ferment melezitose, cellobiose, trehalose or mannose, and thus has a more stable sugar fermentation pattern than *B. thermophilum*, the fermentation patterns of the two species are often the same.

Can develop in 90% air + 10% CO_2 without cells becoming catalase- or catalase-like (hemin)-positive.

Requirements for growth factors are unknown.

Fructose-bisphosphate aldolase and glucose-6-phosphate dehydrogenase present in cell-free extracts as with *B. thermophilum*.

The DNA homology relationships between *B. boum* and *B. thermophilum* have been studied extensively (Scardovi et al., 1979b).

A range of 36–74% is reported for the DNA relatedness of *B. boum* to *B. thermophilum* (Table 15.52); unrelated to any other species in the genus.

Transaldolase isozyme of *B. boum* (number 6) is clearly distinct electrophoretically from that of the majority (93%) of the strains of *B. thermophilum* (number 8).

The PAGE proteins pattern of *B. boum* is distinct and easily distinguishable from that of *B. thermophilum*. The so called "genus band" is slightly less anodal in *B. boum* than in any other species (Biavati et al., 1982).

Interpeptide bridge of the cell wall peptidoglycan is Lys-D-Ser-D-Glu; it differs from that of the closely related species *B. thermophilum* which is Orn(Lys)-D-Glu.

The mol% G + C of the DNA is 60.0 ± 0.2 (T_m).

Type strain: ATCC 27917 (RU917 from bovine rumen, Scardovi et al., 1979).

Comments. The practical distinction of *B. boum* from *B. thermophilum* and *B. choerinum* which are often found in the same ecological niches, can be achieved with transaldolase electrophoresis or with PAGE proteins electrophoresis (see *Comments* under *B. choerinum*).

17. Bifidobacterium magnum Scardovi and Zani 1974, 31.[AL]

mag'num L. adj. *magnus* large, great.

Cells grown in TPY agar are usually characteristically long and thick, with irregular contours, measuring 2 × 10–20 μm and occurring frequently in aggregates (Fig. 15.97*D*).

Anaerobic. CO_2 without effect on O_2 sensitivity or anaerobic growth.

Sparse growth in TPY medium not containing Tween 80, as Tween 80 is highly stimulatory (Scardovi and Zani, 1974).

The unique acidophilic species of the genus. Its original optimum pH for growth is 5.3–5.5; growth is retarded at 5.0 or at 5.9; no growth (after 2 days) at 4.2 or 7.0.

Fructose-bisphosphate aldolase present in cell-free extracts (4–5 mU/mg of proteins); $NADP^+$-dependent glucose-6-phosphate dehydro-

genase demonstrable. Aldolase spot-stainable on electrophoresis (Scardovi and Sgorbati, 1974).

The isozyme pattern 5-7 (transaldolase and 6PGD types resp.) is unique among bifidobacteria (Scardovi et al., 1979a).

PAGE proteins pattern clearly distinct from that of any other species of the genus (Biavati et al., 1982).

DNA unrelated to that of other species of the genus.

Found in the feces of rabbit.

The mol% G + C of the DNA is 60.0 ± 0.6 (T_m).

Type strain: ATCC 27540 (RA3 from feces of rabbit, Scardovi and Zani, 1974).

Comments. The recognition of *B. magnum* should not be based solely on the unusually large dimensions of its cells. Fermentation of lactose and starch are useful in differentiating *B. magnum* from the "animal" species *B. pullorum*, *B. animalis*, *B. globosum* and *B. pseudolongum*, (which also ferment pentoses), as *B. magnum* ferments lactose but not starch while these other species either ferment both these sugars or neither (*B. pullorum*). *B. magnum* can be distinguished from the human species *B. infantis* and *B. longum* only by virtue of the different 6PGD isozyme present (the transaldolase isozyme of *B. magnum* is shared by *B. infantis*, number 5). Alternatively, PAGE procedure can be used.

18. **Bifidobacterium pullorum** Trovatelli, Crociani, Pedinotti and Scardovi 1974, 197.[AL]

pul'lus. L. n. *pullus* a chicken; L. pl. gen. n. *pullorum* of chicken.

Cells grown in TPY agar stabs are slightly curved, 2–8 μm long, with tapered ends, mostly arranged in irregular chains often of great length (Fig. 15.97*A*). Cells are frequently poorly refractile and appear to be empty or vacuolized. Branchings are rare.

Anaerobic. CO_2 without effect on O_2 sensitivity or anaerobic growth.

Requires nicotinic acid, pyridoxine, thiamin, folic acid, *p*-aminobenzoic acid and Tween 80 for satisfactory growth.

Fructose-bisphosphate aldolase present in cell-free extracts in considerable amounts (20–30 mU/mg proteins). As with most *B. dentium* strains, neither 6PGD ($NADP^+$ or NAD^+ dependent) nor glucose-6-P-dehydrogenase can be detected.

Lactic and acetic acids are produced in a ratio of 1:3.5 ± 0.2 in TPY medium but unlike all other species of the genus, the isomeric type of lactic acid formed is DL.

The mol% G + C of the DNA is 67.4 ± 0.4 (T_m), the highest value so far found in bifidobacteria.

The DNA is not related to that of any of the other species.

Found in feces of chicken.

Type strain: ATCC 27685 (P145 from feces of chicken, Trovatelli et al., 1974).

Comments. Morphology is of help in recognizing this species. Furthermore, it can be easily distinguished from the "animal" species of the genus which also ferment arabinose, xylose and ribose because it does not ferment lactose and starch (see *Comments* under *B. magnum*). Can be distinguished from other species of the genus on the basis of the fermentative characters (Table 15.55).

19. **Bifidobacterium suis** Matteuzzi, Crociani, Zani and Trovatelli 1971, 393.[AL]

su'is. L. n. *sus* pig; L. sing. gen. n. *suis* of pig.

Cells grown in TPY agar stabs show a similar morphology to those of many other species of the genus (Fig. 15.98*D*).

Anaerobic. CO_2 without effect on O_2 sensitivity or anaerobic growth.

Riboflavin is the only growth factor required.

Cell-free extracts possess fructose-bisphosphate aldolase and HMP dehyrogenases. Most strains possess a constitutive urease activity (not influenced by urea or organic nitrogen sources). Fifty percent of the strains studied share with a percent of strains *B. longum* and *B. infantis* the isozyme electrophoretic pattern transaldolase 6 and 6PGD 5; the others display a pattern (6-8) which is common in *B. longum* (Scardovi et al., 1979a). PAGE proteins pattern is quite distinct from that of the other species of the genus (Biavati et al., 1982).

The mol% G + C of DNA is 62 (T_m).

Unrelated in DNA homology to any other species of the genus.

So far, found only in the feces of piglets.

Type strain: ATCC 27533 (Su859 from feces of pig, Mateuzzi et al., 1971).

Comments: B. suis can be distinguished readily from other bifid species commonly found in the pig, namely *B. globosum*, *B. pseudolongum*, *B. thermophilum*, *B. boum* and *B. choerinum*, on the basis of fermentative characters: the last three species do not ferment arabinose and xylose whereas *B. suis* does; the first two ferment starch whereas *B. suis* does not (see Tables 15.51 and 15.54; Zani et al., 1974).

20. **Bifidobacterium minimum** Biavati, Scardovi and Moore 1982, 368.[VP] This taxon was previously described and referred to as "*minimum*" DNA homology group, and consists at present only of two strains isolated from sewage (Scardovi and Trovatelli, 1974).

min' i.mum. L. adj., least; *minimum* the least.

Cells grown in TPY agar stabs are characteristically very small (0.3 × 1.3–1.5 μm) with tapered ends; sometimes irregularly branched (Fig. 15.97*F*). This morphology resembles that *B. asteroides*, but star-like aggregates, characteristic for that species, are absent here.

Anaerobic. CO_2 without effect on O_2 sensitivity or anaerobic growth.

Sugars fermented include glucose, fructose, sucrose, maltose and starch.

The interpeptide bridge of the cell wall peptidoglycan is Lys-Ser, unique among bifidobacteria.

Cells possess the least anodal form of transaldolase among bifidobacteria (No. 10, i.e. migrating 66, whereas the most anodal isozyme, No. 1, in *B. cuniculi*, migrates 100) (Scardovi et al., 1979a).

Aldolase and glucose-6-phosphate dehydrogenase not detected.

Distinct PAGE proteins pattern.

DNA unrelated to that of any other species of the genus.

Found until now in a single specimen of waste water.

The mol% G + C of the DNA is 61.5 (T_m).

Type strain: ATCC 27538 (F392 from waste water, Scardovi and Trovatelli, 1974).

21. **Bifidobacterium subtile** Biavati, Scardovi and Moore 1982, 36[VP] This taxon was previously described and referred to as "*subtile*" DNA homology group and includes five strains isolated from sewage (Scardovi and Trovatelli, 1974).

sub'ti.le. L. adj. slender, *subtile* the slender.

Cells grown in TPY agar stabs are slender, 0.5 × 2–3 μm with rounded or tapered ends, sometimes curved; branchings are rare. This morphology (Fig. 15.97*E*) is similar to that of *B. breve*, but the cells of the latter are usually shorter and thicker, swollen and branched.

Anaerobic. CO_2 without effect on O_2 sensitivity or anaerobic growth.

Optimum temperature for growth is in the range of 34–35.5°C, being markedly lower than the range 37–41°C, valid for the other species of the genus.

Sugars fermented are similar to those fermented by *B. breve* but, unlike *B. breve*, lactose is not fermented (Table 15.55); also *B. subtile* ferments starch and gluconate whereas *B. breve* does not.

Possesses high levels (10–15 mU/mg proteins, in strain F395) of $NADP^+$-NAD^+-dependent glucose-6-phosphate dehydrogenase but aldolase is not measurable.

Transaldolase and 6PGD isozymes are among the most anodal in the bifidobacteria (number 3 and 2, respectively); this 3-2 pattern occurs only in this species (Scardovi et al., 1979a).

Distinct PAGE proteins pattern.

Both human and animal types of F6PPK have been detected in cell-free extracts (Scardovi and Trovatelli, 1974).

Unrelated by DNA homology to any other species of the genus.

The mol% G + C of the DNA is 65.5 (T_m).

As yet isolated only from two specimens of waste waters.

Type strain: ATCC 27537 (F395 from waste water, Scardovi and Trovatelli, 1974).

22. **Bifidobacterium coryneforme** (ex Scardovi and Trovatelli,

1969) Biavati, Scardovi and Moore, 1982, 368.[VP] *Bifidobacterium coryneforme* Scardovi and Trovatelli (1969) was not included in the Approved Lists (1980). On the basis of its different electrophoretic pattern of cellular proteins and the previously confirmed differential characters, its revival was proposed (Biavati et al., 1982).

co.ry.ne.for'me. Gr. n. *coryne* a club; L. n. *forma* shape, form; M.L. adj. *coryneformis* club shaped.

The strains assigned to this species had been isolated occasionally from the intestine of honey bee *Apis mellifera* subsp. *mellifera* and subsp. *caucasica*, and received from Germany (Bayern), Norway (Billingstad), England (Buckfast) and Bulgaria.

The strains grow poorly in TPY medium but profusely in MRS medium. Cells grown in MRS agar stabs are short (1.1.5 μm long) often lanceolate, single or in pairs, sometimes with short branchings or simple knobs (Fig. 15.98C). Radial groupings of cells are very rare and formed under extreme conditions of growth (Scardovi and Trovatelli, 1969).

Anaerobic. Does not develop in slants inoculated under 90% air + 10% CO_2. CO_2 does not influence anaerobic growth either on solid or in liquid medium.

Aldolase in cell-free extracts not detected. HMP dehydrogenases present.

Transaldolase and 6PGD isozymes pattern (6-6) is shared by some ains of *B. breve*. However zymograms of G3PD (3-phosphoglyceraldehyde dehydrogenase) are distinct (Scardovi et al., 1979a) and the GE proteins pattern is distinct (Biavati et al., 1982).

Unrelated by DNA homology to any other species of the genus except *B. indicum*: an interspecific DNA relatedness of 60% is reported (Scardovi et al., 1970).

Type strain: ATCC 25911 (C215 from intestine of *Apis mellifera* subsp. *caucasica* from Norway (Scardovi and Trovatelli, 1969)).

23. Bifidobacterium asteroides Scardovi and Trovatelli 1969, 83.[AL]

as.te.roi'des. Gr. adj. *asteroides* starlike.

Cells grown in TPY agar stabs are 2–2.5 μm long, generally in pairs with pointed ends and slightly curved; usually in radial disposition around a common mass of hold-fast material (Fig. 15.98A). Nutritional or CO_2 deficiences or growth with certain sugars, may induce clavate or spatulate cells with occasional swellings, irregular or cross-like branchings in the central part of the cell body.

Colonies are circular, smooth, convex, with entire edge; consistency is such that the colony is removed by needle and can hardly be dispersed in water.

Growth in static fluid culture generally adheres to the glass walls and leaves the liquid clear.

is required for growth in all media including stabs.

Aerobic growth occurs on slopes only if air is enriched with 10% CO_2.

boflavin, pantothenate, nicotinic acid, pyridoxine, thiamin and biotin are required for continued growth in a Bacto Vitamin Free Casamino Acids-containing substrate (Scardovi and Trovatelli, 1969).

Temperature relations: optimum 35–36°C; no growth at 21°C or at 42°C (after 7 days incubation).

H_2O_2 is decomposed vigorously by cells grown in 90% air + 10% CO_2. In stabs, incubated in CO_2-air, only the lower portions of the growth are catalase-negative.

Fructose-bisphosphate aldolase is not detected in cell-free extracts or in iodoacetate-poisoned cell suspensions; cell-free extracts possess both the HMP dehydrogenases $NADP^+$-dependent.

A total of 85 strains of very different geographical origins were examined electrophoretically for the content of isozymes of transaldolase and 6PGD: at least eight transaldolases and nine 6PDG were detected, each strain exhibiting only one band for each allozyme. *B. asteroides* posesses the least mobile variant of 6PGD (isozyme 13 moves 53, whereas isozyme 1, the most anodal found in *B. pseudocatenulatum*, moves 100) Scardovi et al., 1979a).

Of 224 strains of this species tested for the presence of plasmids, 74 contained a large variety of extrachromosomal elements of diverse molecular weights ranging from 1.2 to 22 MDa (Sgorbati et al., 1982); the functions coded for by these plasmids are still unknown.

DNA is about 30% homologous to DNA from *B. indicum*, another bifid found in the honey bee. Unexpectedly there is 50% homology to *B. choerinum*, a bifid from piglet, otherwise the DNA is unrelated to any other species of the genus.

Found normally in the intestine of western honey bees; occasionally found in the hind-gut of *Apis cerana*, an asiatic honey bee.

The mol% G + C of the DNA is 59 (T_m).

Type strain: ATCC 25910 (C51 from intestine of *A. mellifera* var. *ligustica* from Italy (Scardovi and Trovatelli, 1969)).

24. Bifidobacterium indicum Scardovi and Trovatelli 1969, 84.[AL]

in'di.cum. M.L. *neut.* adj. *indicum* from specific epithet of bee *Apis indica* F.

Cells grown in TPY agar stabs are generally short, in pairs, often in angular disposition, more or less globular, sometimes suggesting a minute morphovar of *B. globosum* (Fig. 15.98B): other strains have slender and longer cells; star-like clusters of cells never occur. Cells grown on gluconate are extremely small and regular in shape (Sgorbati et al., 1970).

Colonies do not have the consistency of those of *B. asteroides*.

Growth in liquid media does not adhere to the walls; the cells sediment very slowly.

Oxygen tolerance similar to that of *B. asteroides*. CO_2 is required for aerobic growth, whereas for anaerobic growth the effect of CO_2 is equivocal.

H_2O_2 is decomposed only by cells grown in 90% air + 10% CO_2 in the presence of hemin.

Cell-free extracts are fructose-bisphosphate aldolase-negative, but possess the HMP dehydrogenases: glucose-6-P dehydrogenase is both $NADP^+$- and NAD^+-dependent, whereas only $NADP^+$ is effective in 6-phosphogluconate dehydrogenation (Scardovi and Trovatelli, 1969).

A total of 122 strains from different sources (Malaysia, Japan, Philippines) were examined for their transaldolase and 6PGD zymograms: starch gel electrophoresis showed the presence of isozymes Nos. 4 and 7, respectively. Most strains isolated from *A. dorsata* possess a transaldolase isozyme different (number 7) from that found in strains isolated from *A. cerana* (*A. indica*) (number 9) (Scardovi et al., 1979a).

Of a total of 106 strains surveyed for the presence of plasmids, 73 were found to harbor extrachromosomal elements; 57% of these strains had a 22 MDa plasmid while 33% showed a two-banded pattern at 2.0 and 3.5 MDa (Sgorbati et al., 1982). The cellular functions coded for by these plasmids are still unknown.

Unrelated by DNA homology to any other species of the genus.

The mol% G + C of the DNA is 60 (T_m).

Type strain: ATCC 25912 (C410 from intestine of *Apis cerana* (*A. indica*) from Malaysia (Scardovi and Trovatelli, 1969)).